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I. THE USE OF COOKED MEAT MEDIUM FOR THE DETECTION OF C. TETANI

By IDA A. BENGTSON

II. STUDIES ON THE POTENCY TESTING OF PNEUMOCOCCUS VACCINES

By IDA A. BENGTSON

III. THE ADAPTABILITY OF VARIOUS AMERICAN PEPTONES FOR USE IN CHOLERA MEDIA

By IDA A. BENGTSON

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I. THE USE OF COOKED MEAT MEDIUM FOR THE DETECTION OF C. TETANI.[1]

By IDA A. BENGTSON, Associate Bacteriologist, Hygienic Laboratory, United States Public Health Service.

INTRODUCTION.

A study was undertaken to determine the value of cooked meat medium as compared with beef infusion broth in fermentation tubes for the detection of *C. tetani*.

Meat infusion broth in Smith fermentation tubes is the routine medium used for the testing of biologic products in the Hygienic Laboratory. The broth as used in fermentation tubes is adapted for the detection of organisms growing both aerobically and anaerobically. Just prior to use 1 cubic centimeter of a 1 per cent solution of glucose is added to each fermentation tube [2] which contains about 30 c. c. of broth, the tube then being heated in the Arnold sterilizer for a period of 30 minutes, and immediately tipped on removal from the sterilizer to expel the bubble of air. The closed arm affords suitable conditions for the growth of anaerobes, while aerobes may develop in the open arm.

The success attending the use of cooked meat medium in work undertaken by the writer in connection with the study of other anaerobes (*C. botulinum* and *C. parabotulinum*) suggested the possible superiority of the cooked meat medium over broth in fermentation tubes for the detection of *C. tetani*.

The cooked meat medium as used varies slightly from that described by Holman.[3] The history of the use of tissue in media for enhancing the growth of organisms from the time when first used by Theobald Smith (1890) has been reviewed by Holman.

The medium used by the writer is prepared as follows: Beef muscle is ground in a meat grinder and weighed. Two parts of water are added to one part of ground meat. The mixture is cooked in the Arnold sterilizer 1 hour, stirring occasionally. It is then removed from the sterilizer, filtered through filter paper, and allowed to drain for 1 hour. Both the meat residue and the filtrate are saved. The broth filtrate is adjusted to a reaction of pH 8.5. About 5 grams of meat are placed in each tube and 8–10 cubic centimeters of broth added. The tubes are then sterilized in the autoclave at 15 pounds

[1] Manuscript submitted for publication Feb. 25, 1924.

[2] This is for the purpose of enhancing the growth of *C. tetani*, the object being to add only a sufficient amount of glucose to provide a stimulus for growth, and at the same time not enough to bring about excessive acid production which is deleterious to the growth of *C. tetani* and the production of toxin.

[3] Jour. Bact., 1919, 4, 149–155.

pressure for 1½ hours. The following day the medium is heated in the Arnold sterilizer for a period of 1½ hours. Prior to this heating a cap of petrolatum (petroleum jelly, vaseline) about one-eight inch thick may have been added if desired. If the petrolatum cap is added at this time no subsequent heating is necessary. If the medium has been allowed to stand without the petrolatum cap before using, it is advisable to heat for one-half hour just prior to use in order to provide better anaerobic conditions. The reaction of the medium after sterilization and heating is about pH 6.8–7, which reaction seems to be retained even on further heating.

Infusion broth which has been found favorable for the growth of *C. tetani* is of a rich golden brown color. This apparently is a point of considerable importance, since light colored broths (pale straw color) have been found to be much less favorable.[4] The essential point seems to be that the meat infusion and subsequent processes should be carried out in such a way as to extract the greatest amount of substance from the meat. A reaction in the neighborhood of pH 7.6 has been used in the preparation of the various broths.

EXPERIMENTAL WORK.

Various methods of preparing the broths were first investigated.

The following table (Table I) shows the results obtained with broth in fermentation tubes prepared in two different ways. Media B_1 and B_3 were made by infusing the ground meat at refrigerator temperature for 24 hours without squeezing out the juice or prolonged draining; media B_2 and B_4 were prepared by cooking the meat instead of infusing, to extract the juice, the meat having been cut into pieces instead of being ground. Media B_1 and B_2 were made with Parke, Davis & Co.'s crystalline peptone and media B_3 and B_4 with Parke, Davis & Co.'s powdered peptone. Three different sets of tetanus spores were used—Nos. 28 and 29, which had been stored at a temperature of about 10° C. for a period of about 10 years, and No. 253, recently prepared spores suspended in salt solution.

TABLE I.—*Comparison of methods of preparing broth.*

[+=growth in closed arm; g=gas; b=small bubble of gas; superior figures indicate days on which growth and gas appeared.]

	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
MEDIUM BROTH B_1.						
Spores 28	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	----------
	$+^{1}$	$+^{1}g^{2}$	$+^{2}$	$+^{2}g^{2}$	$+^{2}g^{2}$	----------
Spores 29	$+^{2}g^{5}$	$+^{2}g^{2}$	$+^{2}g^{2}$	$+^{2}g^{4}$	----------	----------
	$+^{1}g^{4}$	$+^{2}g^{2}$	$+^{2}$	$+^{2}g^{2}$	----------	----------
Spores 253	$+^{1}b^{2}$	$+^{1}g^{2}$	$+^{1}g^{1}$	$+^{1}$	$+^{1}g^{2}$	$+^{1}$
	$+^{1}g^{1}$	$+^{1}g^{6}$	$+^{1}$	$+^{1}$	$+^{1}$	----------
	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{7}$	$+^{1}$	----------
	$+^{1}g^{2}$	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$	----------

[4] An increase in the brownish color may, on the other hand, be due to overheating, and this should be avoided.

TABLE I.—*Comparison of methods of preparing broth*—Continued.

	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
MEDIUM BROTH B_2.						
Spores 28	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
Spores 29	$+^1g^1$	$+^2g^2$	$+^2g^2$			
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1$	$+^1b^2$	$+^1$	$+^1$	$+^1$	
	$+^1g^2$	$+^1g^2$	$+^1g^1$	$+^1$	$+^1$	
	$+^1g^1$	$+^1g^5$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$
MEDIUM BROTH B_3.						
Spores 28	$+^1$	$+^1g^2$	$+^2g^2$	$+^2$	$+^2g^6$	
	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
Spores 29	$+^1$	$+^2g^2$	$+^2$	$+^2g^2$		
	$+^2g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1g^2$	$+^1g^2$	$+^1$	$+^1$	$+^2$	
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$
	$+^1g^2$	$+^1$	$+^1g^4$	$+^1$	$+^1$	$+^1g^2$
MEDIUM BROTH B_4.						
Spores 28	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	
Spores 29	$+^1g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$	
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		
Spores 253	$+^1g^2$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$
	$+^1g^2$	$+^1g^1$	$+^1g^1$	$+^1$	$+^1g^2$	
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^4$	$+^1g^2$	
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$	

The results may be summarized thus:

	Number planted.	Number grew.	Per cent of growth.
Medium B_1 (meat infused without pressing out)	48	39	
Medium B_3 (meat infused without pressing out)	42	36	
	90	75	83.33
Medium B_2 (meat cooked in the liquid)	48	41	
Medium B_4 (meat cooked in the liquid)	48	40	
	96	81	84.38

The results show about equally good results with the two media prepared according to the two different methods.

Three other sets of broth were prepared, broth C being made with meat cut in pieces and cooked under 15 pounds pressure for 1 hour and allowed to stand over night before straining, broth D, with ground meat infused at ice-box temperature (8–10° C.) for 21 hours, strained and heated in streaming steam for 1 hour, and broth E made with ground meat heated to 70° C. for one-half hour in a double boiler and allowed to infuse for 20 hours at room temperature, then strained and heated in streaming steam for 1 hour. Spores 253 in dilutions at closer intervals than those used in the previous test were planted in the various broths and also in cooked meat medium. The following table (Table II) shows the results obtained:

TABLE II.—*Comparison of methods of preparing broth.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Broth C. (Cooked-meat broth, pH 7.5.)											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$					$+^2g^2$			
$+^1g^2$	$+^2g^2$	$+^2g^2$		$+^2g^3$			$+^2g^2$				
$+^1g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$								
$+^1g^2$	$+^1g^2$	$+^1g^3$	$+^2g^2$	$+^2g^2$	$+^3g^3$	$+^3g^3$					
$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$					
Broth D. (Meat infusion; ice-box, pH 7.4.)											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$							
$+^2g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$							
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$							
$+^1g^2$	$+^1g^2$	$+^1g^3$		$+^2g^2$							
$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^2g^2$	$+^2g^3$					
Broth E. (Meat infusion; heated to 70° C., then infused at room temperature, pH 7.4.)											
$+^1g^2$	$+^1g^3$	$+^2g^2$	$+^2g^3$								
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$			$+^1g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^4$								
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2$	$+^3g^3$	$+^3g^3$						
$+^2g^6$	$+^2g^2$	$+^2g^3$	$+^1g^2$	$+^1g^1$	$+^2g^3$						
Cooked meat medium No. 15.											
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$		$+^2g^2$			$+^2g^2$		
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$						
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$				
$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$		$+^2g^2$		$+^1g^2$		

The results may be summarized thus:

	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Broth C	60	27	45.00
Broth D	60	25	41.66
Broth E	60	26	43.33
	180	78	43.33
Cooked meat medium	60	37	61.11

Here again there was very little difference in the results obtained in the three broths. The reaction of all was pH 7.4 or pH 7.5 and all were of a golden brown color. The results in the cooked meat medium were better than in any of the broths, as indicated by the figures 43.33 per cent of growth in the broth media and 61.11 per cent in the cooked meat medium. The highest dilution in which growth

occurred in the broth was 1:50,000,000, and in the cooked meat medium 1:100,000,000. The highest dilution in which growth occurred in all the tubes planted was 1:500,000 in the case of the broth and 1:2,000,000 in the case of the cooked meat medium.

The effect of standing after heating was tested on several different broths. It is to be expected that on long standing a certain amount of air will be absorbed by the medium and the conditions will be less favorable for the growth of anaerobic organisms. However, there was no marked difference in the growth obtained up to four hours. This is shown by the following test (Table III) which is one of several tests carried out:

TABLE III.—*Effect of length of time broth is left standing after heating.*

BROTH A_2.

Time of planting.	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
12 m	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	----------
1 p. m	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^3$	----------
2 p. m	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$	----------	----------
3 p. m	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^1g^2$	----------	----------
4 p. m	$+^1g^2$	$+^2g^7$	$+^2g^2$	$+^2g^7$	$+^2g^3$	----------

That tetanus spores develop over a considerable range or reaction in broth which is in other respects a suitable one for the organisms is shown by the following test (Table IV). The reaction of the medium was originally pH 7.6. This was adjusted to the reactions indicated by the addition of N/1 NaOH or N/1 HCl.

TABLE IV.—*Effect of reaction.*

BROTH A_4.

pH.	Dilutions of spores.					
	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/10,000,000
7.2	$+^1g^2$	$+^1g^1$	$+^1g^2$	$+^1$--	----------	$+^2g^2$
7.5	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^2g^2$	$+^2g^2$
8.0	$+^1g^1$	$+^1g^1$	$+^1g^1$	$+^1g^2$	----------	$+^1g^2$
8.5	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	----------
Unadjusted (pH 7.6)	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$

A broth which, on the other hand, was not very favorable for the growth of the organisms due to improper methods of preparing the infusion (usually indicated by a pale color) was no more suitable at reactions which varied from the original reaction (pH 7.6) as widely as in the test shown above, than at the original reactions.

A test was put on to determine whether by the addition of one-tenth of 1 per cent of cystine, or a fragment of meat, to the broth, as

good results could be obtained as were obtained in the cooked meat medium. Broths B_1, B_2, B_3, and B_4 were used in the test. The results are shown in the following table (Table V):

TABLE V.—*Effect of adding cystine or glucose to broth.*

	Dilutions of spores.											
	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000	1/2,000,000,000	1/5,000,000,000
0.1 PER CENT CYSTINE ADDED.												
Media B_1	$+^1$	$+^1$	$+^2$									
B_2	$+^1$	$+^1g^2$	$+^2$	$+^2$								
B_3	$+^2$	$+^1g^2$										
B_4	$+^1$	$+^1$	$+^2$	$+^2$								
FRAGMENT OF MEAT ADDED.												
Media B^1	$+^2g^3$	$+^1g^3$			$+^2g^3$			$+^2g^3$				
B^2	$+^2g^3$	$+^2g^2$		$+^2g^4$	$+^2$							
B^3	$+^2g^2$		$+^2$			$+^1$						
B^4	$+^2g^2$	$+^2g^2$					$+^1$			$+^2$		
NO MEAT OR CYSTINE ADDED.												
Media B^1		$+^1$										
B^2	$+^1$	$+^1g^2$			$+^1g^2$							
B^3												
B^4	$+^2$											
COOKED MEAT MEDIUM												
No. 5	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$		$+^2g^2$						
No. 6	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$						
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$		$+^1g^2$						
	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$								
	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$					
	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$	$+^1g^2$							
	$+^1g^1$	$+^1g^1$	$+^1g^2$	$+^1g^2$	$+^1g^2$							

The results obtained may be summarized thus:

Media.	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Broth+0.1 per cent cystine	48	13	27.08
Broth+fragment of meat	48	15	31.25
Broth without addition of cystine or meat	48	5	10.42
Cooked meat	108	51	47.2

Though cystine or meat added to the broth made conditions more favorable for the growth of *C. tetani*, better growth was obtained in the cooked meat than in any of the broths.

The comparative tests of broth and the cooked meat medium as indicated in the preceding table and in Table II are examples of a number of similar tests, in all of which growth was obtained consistently in higher dilutions in the cooked meat medium than in the fermentation tubes containing broth.

In the preceding tests in which the cooked meat medium has been used the petrolatum cap was added before inoculation. Inoculation through the petrolatum cap is, however, inconvenient and requires considerable time in planting. A test was put on to determine the value of the petrolatum cap, with the following results:

TABLE VI.—*Value of petrolatum cap on cooked meat medium.*

Dilutions of spores.									
1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000
No petrolatum cap; spore suspensions planted by allowing them to drip from pipette without touching the medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{3}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$	$+^{1}g^{2}$ ---	--- ---	$+^{3}g^{3}$ ---	--- ---	--- ---	--- ---
No petrolatum cap; spore suspension planted by lowering pipette into medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$	--- $+^{2}g^{2}$	--- ---	$+^{2}g^{2}$ ---	--- ---	--- ---	--- ---
Petrolatum cap; spores suspensions planted by lowering pipette into medium.									
$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$	$+^{2}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ ---	$+^{2}g^{2}$ $+^{2}g^{2}$	--- ---	--- ---	--- ---

The results may be summarized thus:

	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
No petrolatum cap (pipette not lowered into medium)	20	10	50
No petrolatum cap (pipette lowered into medium)	20	10	50
Petrolatum cap	20	13	65

Better results were therefore obtained with the vaseline cap than without.

A trial was made to determine whether as good results could be obtained by adding the petrolatum after planting as with medium to which a petrolatum cap had been added before inoculation. The following table (Table VII) shows the results:

TABLE VII.—*Effect of adding petrolatum to cooked meat medium before and after planting.*

	Dilutions of spores.											
	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000	1/1,000,000,000	1/2,000,000,000	1/5,000,000,000
	PETROLATUM ADDED BEFORE PLANTING.											
No. 11	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$	$^2g^4$							
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$								
No. 12	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$		$+^4g^4$						
	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^4$								
	$+^2g^2$	$+^2$	$+^2g^2$	$+^2g^2$	$+^2g^4$							
	$+^2g^4$	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^4$						
	$+^2g^2$	$+^2g^4$	$+^2g^2$	$+^2g^4$	$+^2g^2$							
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^2$								
	PETROLATUM ADDED AFTER PLANTING.											
No. 11	$+^2g^2$	$+^2g^2$	$+^4g^4$									
	$+^2g^2$	$+^2g^2$	$+^4g^4$	$+^2g^2$	$+^2g^4$		$+^2g^2$	$+^4g^4$				
No. 12	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$		$+^2g^2$						
	$+^2g^2$	$+^2g^2$	$+^2g^4$	$+^2g^2$			$+^4g^4$					
	$+^2g^2$	$+^2g^2$	$+^2g^2$	$+^2g^5$								
	$+^2g^4$	$+^2g^4$	$+^4g^4$			$+^2g^2$						
	$+^2g^2$	$+^2g^2$		$+^2g^2$								
	$+^2g^2$	$+^2g^4$	$+^2g^2$	(a)								

[a] Tube broke.

The results may be summarized thus:

	Number of tubes inoculated.	Number of tubes which grew.	Per cent of growth.
Petrolatum added before planting	96	38	39.5
Petrolatum added after planting	96	34	35.41

The results are slightly in favor of adding the petrolatum before planting.

Another test was put on in which a comparison was made of the cooked meat medium without a petrolatum cap, of cooked meat medium to which the petrolatum cap was added after planting, and of three different lots of broth. The results are shown in the following table:

TABLE VIII.—*Comparison of cooked meat medium with and without petrolatum, and broth.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Cooked meat medium—no petrolatum added.											
$+^2g^2$ $+^1g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ ---	--- $+^2g^2$ $+^2g^2$	--- $+^2g^2$ ---	--- --- ---	$+^2g^2$ --- ---	--- --- ---	--- --- ---	--- --- ---
Cooked meat medium. Petrolatum added after planting.											
$+^1g^2$ $+^1g^2$ $+^1g^2$	$+^1g^2$ $+^2g^2$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^2g^2$	$+^2g^3$ $+^3g^3$ $+^2g^2$	$+^2g^2$ $+^2g^2$ $+^3g^3$	$+^3g^3$ $+^3g^3$ $+^2g^2$	$+^3g^3$ $+^2g^2$ $+^3g^3$	--- $+^3g^3$ $+^3g^3$	--- --- ---	--- --- ---	--- --- ---	$+^3$ --- ---
Broth E.											
$+^1b^2$ $+^1$ $+^1g^2$	$+^1b^2$ $+^1$ $+^1$	$+^1$ --- $+^2$	--- $+^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- $+^2g^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---
Broth D.											
$+^1g^2$ $+^1g^2$ $+^1g^2$	$+^2$ $+^1g^2$ $+^1g^2$	$+^1g^2$ $+^1g^2$ ---	$+^1g^2$ $+^1b^2g^3$ $+^2g^2$	--- $+^1g^2$ ---	--- --- ---	--- $+^2g^2$ ---	--- $+^2g^2$ ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---
Broth C.											
$+^1g^2$ $+^1b^2g^3$ $+^2$	$+^2g^2$ --- $+^2b^2g^3$	--- --- ---	$+^1g^2$ --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---	--- --- ---

The results may be summarized thus:

Media.	Number of tubes planted.	Number of tubes which grew.	Per cent of growth.
Cooked meat medium (no petrolatum added)	36	18	50.00
Cooked meat medium (petrolatum added after planting)	36	23	63.88
Broth E	36	10	27.77
Broth D	36	14	38.88
Broth C	36	6	16.66

The results with the cooked meat medium were considerably better than with the broth. The best results were obtained with the cooked meat medium to which petrolatum was added after planting. Though fairly good growth was obtained with the cooked meat media without petrolatum, in some of the tubes it was difficult to determine whether growth had taken place since the medium was not turbid. While theoretically the meat tubes without petrolatum would offer the best conditions for growth of both aerobes and anaerobes, the

medium would not be practical for the detection of *C. tetani* since in many cases it would be difficult to decide whether growth and gas had been produced or not.

The value of cooked meat medium for the detection of *C. tetani* was tested in two samples of vaccine virus 27 L and 29 H which had been rejected for human use.

Vaccine virus 29 H was inoculated in 0.01 cubic centimeter quantities in the cooked meat medium and three lots of broth. One set of tubes was inoculated with the uncontaminated virus and another with virus contaminated with dilutions of tetanus spores ranging from 1/100,000 to 1/500,000,000. The following table (Table IX) shows growth and gas production in the meat medium, and growth in the closed arm and gas production in the broth.

TABLE IX.—*Growth of C. tetani in vaccine virus.*

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI*.											
$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{3}g^{3}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{2}$ $+^{1}g^{2}$	$+^{1}g^{2}$ $+^{1}g^{3}$ $+^{1}g^{4}$	$+^{1}g^{2}$ --- $+^{2}g^{2}$	$+^{2}g^{2}$ $+^{2}g^{2}$ $+^{2}g^{7}$	$+^{1}$ $+^{2}$ $+^{2}$	$+^{3}$ $+^{1}g^{1}$ ---	--- $+^{1}g^{2}$ $+^{1}$	$+^{1}$ --- $+^{1}g^{4}$
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.											
$+^{2}$ $+^{2}$ $+^{2}$	$+^{2}$ $+^{1}$ ---	--- --- $+^{1}$	$+^{2}$ --- ---	--- --- ---	$+^{2}$ --- $+^{2}$	--- --- $+^{2}$	$+^{2}$ $+^{1}$ ---	$+^{1}$ --- $+^{1}$	--- --- ---	--- --- ---	$+^{1}$ $+^{1}$ ---
	Dilutions of spores.											
	1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
BROTH PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI*												
Broth E	$+^{1}g^{3}$	$+^{1}g^{3}$	$+^{1}$	$+^{2}g^{3}$	---	---	$+^{2}$	---	---	---	---	$+^{3}$
	$+^{1}g^{3}$	$+^{1}$	$+^{1}g^{2}$	$+^{1}g^{1}$	---	$+^{4}$	---	---	$+^{3}$	---	$+^{3}$	---
Broth D	$+^{1}b^{7}$	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{3}$	---	---	$+^{1}g^{2}$	---	---	---	---	---
	$+^{1}g^{3}$	$+^{1}g^{3}$	$+^{3}g^{3}$	$+^{1}$	$+^{3}g^{3}$	---	---	---	---	---	$+^{1}g^{4}$	---
Broth C	$+^{1}g^{2}$	---	$+^{1}g^{2}$	---	---	---	---	---	---	$+^{3}$	---	---
	$+^{1}g^{2}$	$+^{1}g^{2}$	$+^{1}g^{2}$	---	$+^{1}g^{2}$	$+^{1}g^{2}$	---	---	---	---	---	---
BROTH PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.												
Broth E	---	---	---	---	---	$+^{7}$	---	---	$+^{2}$	---	---	---
	---	---	---	---	---	$+^{3}b^{7}$	---	---	---	---	$+^{2}$	---
Broth D	---	---	---	$+^{1}$	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---	---	---	---
Broth C	---	---	---	$+^{1}$	---	---	---	---	---	---	---	---
	---	---	---	---	---	---	---	---	---	---	---	---

Tests were made on mice with cultures which indicated the presence of *C. tetani* as evidenced by growth in the closed arm of the fermentation tube and by the production of gas in both media. The following table (Table X) shows the results of these tests (+ indicating tetanus in mice; – indicating *C. tetani* absent in tubes as shown by appearance of growth or negative results in mice):

TABLE X.—*Growth of C. tetani in vaccine virus.*

[Tests on mice.]

Dilutions of spores.											
1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*											
+	+	+	+	+	+	+	+	–	–	–	–
+	+	+	+	+	+	–	–	–	–	+	–
+	+	+	+	+	+	+	–	–	–	–	–
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.											
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–
BROTH PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*											
	Dilutions of spores.										
	1/100,000	1/200,000	1/500,000	1/1,000,000	1/2,000,000	1/5,000,000	1/10,000,000	1/20,000,000	1/50,000,000	1/100,000,000	1/200,000,000	1/500,000,000
Broth E	+	+	+	+	–	–	–	–	–	–	–	–
Broth D	+	+	+	+	–	–	+	–	–	–	–	–
Broth C	+	+	+	–	+	+	–	–	–	–	–	–
BROTH PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.												
Broth E	–	–	–	–	–	–	–	–	–	–	–	–
Broth D	–	–	–	–	–	–	–	–	–	–	–	–
Broth C	–	–	–	–	–	–	–	–	–	–	–	–

The results may be summarized thus:

Medium.	Number of tubes planted.	Number of tubes in which *C. tetani* grew.	Per cent of growth of *C. tetani*.
Cooked meat medium	36	23	63.88
Broth	72	25	34.72

The highest dilution in which *C. tetani* developed in the cooked meat medium was 1/200,000,000 and in the broth 1/10,000,000. The highest dilution in which *C. tetani* developed in all tubes planted was 1/5,000,000 in the case of the cooked-meat medium and 1/100,000 in the case of the broth.

The other sample of vaccine virus (27 L) contained an organism which produced a large amount of gas in the meat medium. Ninety-six tubes of the meat medium to which petrolatum was added before planting and 96 tubes of meat medium to which petrolatum was added after planting were inoculated with 0.1 cubic centimeter quantities of the vaccine virus contaminated with amounts of tetanus spores ranging from 1/100,000 cubic centimeter to 1/500,000,000 cubic centimeter. Twelve tubes of each set of meat tubes were also planted with 0.1 cubic centimeter quantities of uncontaminated vaccine virus. All tubes (100 per cent) showed growth and gas production after 48 hours incubation. Ninety-six tubes of broth without cystine and 96 tubes of broth containing 0.1 per cent cystine were also planted with spores as above. Fifty-nine of these showed 5 per cent or more of gas in the closed arm, 78 showed a bubble of gas after 3 days incubation. All except 3 showed growth in the closed arm. This vaccine virus was evidently quite heavily contaminated. In the cooked-meat medium it was not possible to distinguish by appearance of the growth or by smears as to the presence of *B. tetani*. No tests on mice were carried out with this set of tubes.

Another test was put on, using the cooked-meat medium, 0.01 cubic centimeter of the same vaccine virus being used for the inoculum. The dilutions of tetanus spores used for contaminating a portion of the vaccine virus ranged from 1/2,000,000 to 1/2,000,000,000. Ten tubes of the meat medium were planted each with 0.01 cubic centimeter of uncontaminated virus. All the tubes showed growth and gas production within 48 hours.

Tests carried out on mice gave the following results (Table XI), 0.1 cubic centimeter of the growth of each tube being inoculated subcutaneously (+ indicating the development of tetanus symptoms). It was necessary to test all the tubes since all showed gas.

TABLE XI.—*Growth of C. tetani in vaccine virus.*

Dilutions of spores.									
1/2,000,000	1/4,000,000	1/10,000,000	1/20,000,000	1/40,000,000	1/100,000,000	1/200,000,000	1/400,000,000	1/1,000,000,000	1/2,000,000,000
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS CONTAMINATED WITH *C. TETANI.*									
+	+	+	+	+	−	−	+	−	−
+	+	+	+	+	−	+	−	−	−
+	+	+	+	−	−	−	−	−	−
COOKED MEAT MEDIUM PLANTED WITH VACCINE VIRUS WITHOUT *C. TETANI* CONTAMINATION.									
−	−	−	−	−	−	−	−	−	−

The results may be summarized thus:

Medium.	Number of tubes planted.	Number of tubes in which *C. tetani* grew.	Per cent of growth of *C. tetani.*
Cooked meat	36	16	44.44

The results show that *C. tetani* grew and toxin was produced in dilutions as high or higher than in tests with pure cultures or with a vaccine virus containing few other organisms. The highest dilution in which growth occurred was 1/400,000,000 and the highest dilution in which growth occurred in all of the tubes inoculated was 1/20,000,000. The presence of other gas-producing organisms did not hinder the development of *C. tetani* or the production of toxin.

SUMMARY AND CONCLUSIONS.

The tables presented show that the cooked meat medium is more sensitive for the detection of *C. tetani* spores in pure culture than is broth in fermentation tubes.

In order to obtain the best results with the cooked meat medium in the detection of anaerobes it is advisable to use a petrolatum cap. This may be added before or after planting. An advantage of adding the petrolatum cap before planting is that further heating of the medium is obviated.

The results as applied to the sterility testing of biologic products indicate that the meat medium with a petrolatum cap is of distinct value in the detection of gas-producing anaerobes. When used with a petrolatum cap, however, conditions are not altogether favorable

for the growth of aerobic organisms, though it is probable that many of the latter are facultative anaerobes and would also develop in media suitable for anaerobes. The cooked meat medium without a petrolatum cap would offer conditions suitable for both aerobes and anaerobes but possibly somewhat at the expense of the anaerobes as compared with the previously capped medium. Gas production also can not be so readily observed since the gas is not confined, and this would probably necessitate the daily observation of the tubes. The growth does not always bring about a distinguishable turbidity and some tubes having growth might be overlooked.

The conclusion may therefore be drawn that the cooked meat medium with a petrolatum cap is useful as an adjunct to broth in fermentation tubes as a means for detecting gas-producing anaerobes in biologic products.

The results of the tests on two different samples of vaccine virus artificially contaminated with *C. tetani* indicate the superiority of the cooked meat medium over the broth for the detection of *C. tetani*, in this menstruum. In the case of one of the samples, few organisms were present, in the other gas-producing organisms were present. In the case of the former *C. tetani* was isolated in 63.88 per cent of cooked meat medium tubes and in 34.72 per cent of the broth tubes. In the latter *C. tetani* was isolated in 44.44 per cent of cooked meat medium tubes. (In this case higher dilutions of spores were used.) The results of both tests indicate that *C. tetani* can be detected in vaccine virus artificially contaminated with *C. tetani* spores in as high dilutions as with pure cultures. The presence of other gas-producing organisms did not hinder the development of *C. tetani* nor the production of toxin.

In addition to the fact that the cooked meat medium is more sensitive for the detection of *C. tetani*, another advantage lies in the fact that gas is always produced. This is not true of broth, a number of tubes planted with pure culture failing to show gas though growth occurred in the closed arm.

II. STUDIES ON THE POTENCY TESTING OF PNEUMOCOCCUS[1] VACCINES.

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INTRODUCTION.

The recent publications of Cecil and Steffen (1921, 1923) describing the successful vaccination of monkeys against pneumococcus pneumonia have served to arouse new interest in the subject of pneumococcus vaccine. The use of this vaccine for prophylaxis in man as practiced by Lister (1913, 1916, 1917) in South Africa and by Cecil and Austin, and Cecil and Vaughan in this country on recruits in the United States Army indicate that the product has a certain value. The results obtained were, however, not as conclusive as might be desired.

Further work by Cecil and his coworkers has served to elucidate some of the problems arising in connection with the use of pneumococcus vaccine. By means of intratracheal inoculations of pneumococcus cultures, Blake and Cecil found it possible to reproduce the clinical picture of human lobar pneumonia in monkeys and thus were able to establish a basis for determining experimentally the value of the vaccine in the prevention of the disease itself.

Cecil and Blake found in their early experiments that subcutaneous inoculations of monkeys with Type I pneumococcus vaccine in doses approximating those which had been employed in man did not afford protection against experimental pneumococcus Type I pneumonia. It was necessary to increase the dose considerably in order to produce immunity by the subcutaneous route as shown by Cecil and Steffen (1921). Intravenous inoculations with comparatively small doses were however successful.

The results obtained in man suggest that a higher degree of immunity would be secured with larger doses of vaccine. The severe reactions which sometimes occur in subcutaneous inoculation even with vaccines as dilute as those employed in the vaccination of soldiers in the United States Army by Cecil and Austin, and Cecil and Vaughan would militate against the use of very concentrated vaccines unless methods are devised for modifying the vaccine or the methods of administration.

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Cecil and Steffen (1922) report the successful immunization of monkeys against Type I pneumonoccus pneumonia by administering vaccine in large or small doses by the intratracheal route, and suggestive results were obtained by spraying the vaccine into the throat.

Perlzweig and Steffen report successful immunization of mice with modified pneumococcus vaccines consisting of the protein fraction obtained either by treating pneumococci with anhydrous sodium sulphate and dissolving the precipitate in dilute sodium carbonate solution or by dissolving pneumococci in bile salts, precipitating with alcohol and dissolving the precipitate in dilute alkaline solutions. Immunization could also be effected by the use of antigens isolated from each of the three fixed types of pneumococcus by tryptic digestion of the pneumococci and extraction of the digest with 70 to 90 per cent alcohol, the degree of immunity conferred being about equal to that obtained with the original saline vaccine.

The results reported above suggest that pneumococcus vaccine may become a prophylactic agent of practical value. This necessitates devising methods for testing the comparative value of such vaccines.

The work here reported was done following the publication of the early papers by Cecil and Austin (1918) and Cecil and Vaughan (1919). It was the purpose to determine the most suitable animal for testing purposes, using vaccines of known composition produced at the Hygienic Laboratory and then to test a number of commercial vaccines. In view of the fact that at the time this work was undertaken not enough information was at hand to decide what a standard vaccine should be, the work done should be regarded as an attempt to carry out certain tests for the purpose of throwing light on the general subject rather than as the definite working out of a specific method. As will be noted later, the commercial vaccines tested were of the greatest variety in their composition and in the dosage recommended.

For test animals rabbits and mice known to be susceptible to pneumococcus infection suggest themselves as being most useful for laboratory testing. As demonstrated by Cecil and Steffen (1921, 1923), monkeys are probably most suitable for determining the value of vaccine for human prophylaxis, but the use of monkeys for the routine testing of a considerable number of vaccines is not practicable.

If the rabbit could be used for the testing of pneumococcus vaccine in the same way as it is used for the testing of typhoid vaccine, i. e., by inoculating the animal and then testing the serum for agglutinins or other antibodies, the matter of testing would be comparatively simple. The use of three sets of animals would be avoided, as the same serum could be tested against all the three types of organisms. The simplicity of carrying out tests in this way, providing it could be shown that such tests were an index of the immunity

conferred, seemed to warrant the investigation of this method at considerable length. Most of the results obtained, however, showed that antibodies in the serum are present in relatively low concentration, and it is doubtful whether their demonstration would give a satisfactory test. In the following discussion some illustrations will be given showing the limitations of this method of testing.

The advantage of a test for active immunity as indicated by immunity against the live organism when inoculated into vaccinated animals is obvious except for the fact as just stated that large numbers of animals are required. Although the test is not the most rigid that might be used, i. e., protection against the disease as it occurs clinically, it is a test against pneumococcus infection, and it is assumed that this is an indication of its effectiveness against the disease itself.

The vaccines made by the Hygienic Laboratory were made with virulent cultures of the three different types of pneumococcus. The cultures were grown in glucose broth for 20–24 hours, after which the organisms were sedimented by centrifugalization. The organisms were suspended in a small amount of salt solution and heated to a temperature of 52–53° C. for one-half hour. The suspension was diluted to 2,500,000,000 or 3,000,000,000 organisms per cubic centimeter, using the Hopkins method of counting. Tricresol in a concentration of 0.25 per cent was used as preservative. In addition to the vaccines prepared thus, several commercial vaccines were used in the preliminary experimental work.

EXPERIMENTS WITH RABBITS.

Type I vaccine.—The tests made on rabbits include tests for active immunity as well as agglutination, precipitin and tropin tests on the serum, and bacteriostatic tests on the whole blood.

Active immunity.—The marked protection afforded in rabbits by three different Type I vaccines is shown in Table I. A series of six rabbits was inoculated intravenously with each of the three vaccines at intervals of 6 to 7 days, a dose of 1 cubic centimeter of the vaccine being injected each time. The various dilutions of live virulent culture were injected intraperitoneally 16 days after the last injection of vaccine.

TABLE I.—*Active immunity in rabbits inoculated intravenously with Type I pneumococcus vaccine.*

	Pneumococcus isolated.
VACCINE A.	
Rabbit 1. 0.00,000,1 cubic centimeter. Survived.	
2. 0.00,001 cubic centimeter. Survived.	
3. 0.00,01 cubic centimeter. Survived.	
4. 0.00,1 cubic centimeter. Survived.	
5. 0.01 cubic centimeter. Survived.	
6. 0.1 cubic centimeter. + 48 hr.	+

Pneumococcus isolated.

VACCINE B.

Rabbit 7. 0.00,000,1 cubic centimeter. Survived.
9. 0.00,001 cubic centimeter. Survived.
10. 0.00,01 cubic centimeter. Survived.
11. 0.00,1 cubic centimeter. Survived.
12. 0.01 cubic centimeter. Survived.

VACCINE C.

Rabbit 13. 0.00,000,1 cubic centimeter. Survived.
14. 0.00,001 cubic centimeter. Survived.
15. 0.00,01 cubic centimeter. Survived.
16. 0.00,1 cubic centimeter. Survived.
17. 0.01 cubic centimeter. Survived.
18. 0.1 cubic centimeter. Survived.

CONTROLS.

Rabbit 19. 0.00,000,1 cubic centimeter. +50 hr. +
20. 0.00,000,01 cubic centimeter +76 hr. +
21. 0.00,000,001 cubic centimeter. Survived.

The table shows that rabbits were protected against 100,000 fatal doses of Type I pneumococcus by vaccine A, against at least 100,000 fatal doses by vaccine B and against at least 1,000,000 fatal doses by vaccine C. The results are definite and pronounced, indicating a high degree of protection by the vaccines used against pneumococcus infection.

Agglutination tests on serums.—Seven days after the last injection of vaccine the rabbits were bled and agglutination tests carried out with the following results (Table II):

TABLE II.—*Agglutination tests on serums of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations). (Tests made 7 days after last inoculation.)*

	Rabbit.	Dilutions of serum.					
		1/1	1/2	1/5	1/10	1/20	1/40
Series I—Vaccine A	1	[1] 2	2	2	2	1	0
	2	2	2	2	2	1	0
	3	2	2	2	2	0	0
	4	3	4	4	4	3	0
	5	3	4	4	4	3	1(?)
	6	1(?)	0	0	0	0	0
Series II—Vaccine B	7	2	2	1	1	0	0
	[2] 8						
	9	3	4	4	4	2	0
	10	3	3	4	3	1	0
	11	3	4	4	4	4	2
	12	1	1	1(?)	0	0	0
Series III—Vaccine C	13	2	3	3	3	1	0
	14	2	2	2	1	0	0
	15	3	3	3	1	0	0
	16	4	4	4	3	3	1
	17	2	2	3	4	3	2
	18	2	2	2	1	0	0
Normal rabbit		0	1	0	0	0	0
Antigen without serum, 0.							

[1] 4 indicates complete agglutination; 3 about 75% agglutination; 2 about 50% agglutination, and 1 about 25% agglutination.
[2] Rabbit died.

About two months after the date of the last inoculation seven rabbits were bled and agglutination tests again carried out with antigen A (vaccine A), with the results shown in Table III. The rabbits had meanwhile received varying small doses of living pneumococcus culture as shown in Table I.

TABLE III.—*Agglutination tests on serums of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations). (Tests made 2 months after last inoculation of vaccine.)*

	Dilutions of serum.					
	1/1	1/2	1/5	1/10	1/20	1/40
Rabbit 1	0	0	0	0	0	0
Rabbit 4	4	3	2	0	0	0
Rabbit 7	1(?)	0	0	0	0	0
Rabbit 11	4	3	2	1	0	0
Rabbit 12	0	0	0	0	0	0
Rabbit 17	4	4	3	1	0	0
Rabbit 18	0	0	0	0	0	0
Normal rabbit	0	1	1	0	0	0

Antigen without serum, 0.

The results obtained in the two tests indicate a rather low agglutinin response. A decrease in the agglutinin titer is shown by the second test. The results in the two tests are consistent in that in general the rabbits which showed the best agglutinin response in the first test continued to do so also in the second test.

The results on the whole however are not very definite. Agglutination was present in only low dilutions and there was considerable variation among the different rabbits. The indications are that the agglutinin response is not sufficient to be demonstrated satisfactorily when three injections of vaccines with the doses employed are made. It is possible with a larger number of injections somewhat higher agglutination titers could be shown.

Bacteriostatic tests.—The use of these tests was suggested by the work of Heist, Solis-Cohen and Solis-Cohen; and Heist and Solis-Cohen. As pointed out by Bull and Bartual the action of whole blood on pneumococci is bacteriostatic rather than bactericidal. As stated by these authors, "cultures of fresh whole blood of immune animals, as compared with cultures in the blood of susceptible animals, show a greatly prolonged latent period, and, in a general way, the relative lengths of the latent periods of the cultures correspond to the relative resistances of the animals to infection by these organisms."

Tests were carried out according to the method of Heist, Solis-Cohen and Solis-Cohen. The test as performed by these authors is essentially as follows: Graded dilutions of an 18-hour broth culture are drawn, up to a fixed point, into sets of five or more capillary pipettes of about 1 millimeter diameter, the culture being immediately withdrawn by touching the end of the pipette to a piece of sterile gauze. A film of culture is thus left on the inner wall of the tube.

After drying for a few minutes in the air, the tube is filled to the fixed point with blood from the rabbit, the end of the pipette being touched to the drop of blood as it flows from the puncture in the ear vein. The capillary tubes are then sealed, incubated and smears made from the contents. The presence of pneumococci in the smear indicates multiplication of the organism. The absence of the organism indicates bactericidal or bacteriostatic action of the blood. As explained by Bull and Bartual, the inhibition of the multiplication of the organisms is dependent on opsonization of the pneumococci by the immune serum and phagocytosis of the organisms by the polynuclear cells. In any case it is necessary to fix certain standards of amounts of culture used, time of incubation, etc. in order to obtain comparable results.

In the tests carried out by the writer, the method of bleeding the rabbit in the afternoon and making smears the following morning was adopted. The following table (Table IV) illustrates the results obtained with Type I pneumococcus in blood from rabbit 17, which had withstood the inoculation of 0.01 cubic centimeter of live culture, and from rabbit 18, which had withstood the inoculation of 0.1 cubic centimeter culture. (See Table I.)

TABLE IV.—*Bacteriostatic tests on blood of rabbits inoculated intravenously with Type I pneumococcus vaccine (3 inoculations).*

[+ indicates pneumococci in smears; − indicates pneumococci not in smears.]

Rabbit.	Dilutions of culture.					
	0.1	0.01	0.00,1	0.00,01	0.00,001	0.00,000,1
No. 17	+	+	−	−	−	−
No. 18	+	+	−	+	−	−
Normal No. 1	+	[1] +	+	+	−	−
Normal No. 2	+	+	+	[1] +	−	−

[1] Few.

The culture used was a virulent one, killing mice on a dose of 0.00,000,001 cubic centimeter. The differences between the vaccinated and normal rabbits are not very marked, though there is a suggestion of a difference between them.

Similar tests were carried out on two United States Army vaccines and several commercial vaccines, tests being made against all three types of organism. The results with two such vaccines are shown in the accompanying tables. (Tables V and VI.) In certain cases the number of injections of vaccine was increased to six. A modification of the technique of Heist, Solis-Cohen and Solis-Cohen was used in several of the tests. This consisted of the use of larger amounts of culture and blood, agglutination tubes being used in place of capillary tubes. A small amount of culture dilution was placed in the tube and a given number of drops of blood were collected into the tube from the ear vein of a rabbit. This method did not, however, afford more satisfactory results than the first method used.

TABLE V.—*Bacteriostatic tests on blood of rabbits inoculated intravenously or subcutaneously with polyvalent pneumococcus vaccine (3 inoculations).*

[Inoculations of vaccine Oct. 25, Nov. 1, Nov. 8. Test Nov. 18.]

	Dilutions of culture.				
	0.1	0.01	0.001	0.00,01	0.00,001
INTRAVENOUS INOCULATION OF VACCINE.					
Rabbit 1a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	+	+	−	−	−
Rabbit 2a:					
Type I	+	+	−	−	−
II	+	−	−	−	−
III	+	−	−	−	−
SUBCUTANEOUS INOCULATION OF VACCINE.					
Rabbit 19a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	−	−	−	−	−
Rabbit 23a:					
Type I	−	−	−	−	−
II	−	−	−	−	−
III	+	−	−	−	−
Control rabbit 1a:					
Type I	+	+	+	+	−
II	+	+	−	+	+
III	+	+	+	+	+
Control rabbit 2a:					
Type I	+	+	+	+	+
II	+	+	+	−	−
III	+	+	+	−	−

TABLE VI.—*Bacteriostatic tests on blood of rabbits inoculated intravenously with a polyvalent commercial vaccine.*

[Inoculations of vaccine Nov. 11, 19, 25, Dec. 2. Test Dec. 11–12.]

	Dilutions of culture.					
	0.1	0.01	0.001	0.0001	0.00,001	0.000,000,1
VACCINATED.						
Rabbit 1b:						
Type I	+	−	−	−	−	−
II	+	+	+	+	−	−
III	+	+	+	+	Few	−
Rabbit 2b:						
Type I	+	+	−	−	−	−
II	+	+	+	+	+	−
III	+	+	+	+	Few	−
Rabbit 6b:						
Type I	+	+	+	Few	+	+
II	+	+	+	+	+	Few
III	+	+	+	+	Few	Few
Rabbit 11b:						
Type I	+	+	Few	Few	−	−
II	+	+	+	Few	+	−
III	+	+	+	+	Few	−
Rabbit 17b:						
Type I	+	+	−	Few	−	−
II	+	+	+	Few	−	−
III	+	+	Few	Few	−	−
Rabbit 4b:						
Type I	+	Few	−	−	−	−
II	Few	+	Few	+	Few	−
III	+	+	+	−	−	−
Rabbit 5b:						
Type I	+	Few	−	−	−	−
II	−	Few	+	−	+	−
III	−	+	Few	−	Few	−
Control rabbits:						
No. 1b	+	+	+	Few	−	−
	+	+	+	+	Few	Few
	+	+	+	+	Few	+
No. 2b	+	+	+	Few	−	−
	+	+	+	+	+	−
	+	+	+	+	Few	−
No. 3b:	+	+	+	+	+	Few
	+	+	+	+	+	+
	+	+	+	+	Few	Few

Regarding the practical value of the test, it may be stated that while the results are suggestive and perhaps in some cases rather definite, still it does not compare in definiteness with the direct protection test.

Precipitin and tropin [2] *tests.*—These tests were carried out on the serums of sets of rabbits inoculated with several different vaccines. In a set of 8 rabbits inoculated intravenously 4 times with a polyvalent commercial vaccine the tropin test was negative on 2 rabbits, and positive on 6 and among these 1 was strongly positive. The precipitin test was found to be positive occasionally in low dilutions of serum but no definite results were obtained. Of these two tests it may be said that in certain cases the results were suggestive and that it might be possible to distinguish between serums from vaccinated and from control rabbits but that for ascertaining the comparative value of different vaccines these two methods were of little value.

Summarizing the results obtained by the various methods used in testing the Type I vaccines, it is obvious that the test for active immunity is by far the most pronounced. The results were so definite that it would decidedly be the method of choice for testing Type I vaccines. A series of 6 rabbits with 2 or 3 control rabbits for determining the virulence of the culture would furnish a satisfactory test for such vaccines.

Type II and III vaccines.—The satisfactory results obtained in the testing of Type I vaccines by the active immunity test on rabbits could not be duplicated with Type II and III vaccines. Although it was possible to show protection against infection with these types in a number of cases, the results were often unsatisfactory for the reason that the virulence of these cultures for rabbits fluctuated very greatly or was very low. The cultures of all three types were almost invariably fatal to mice in dilutions of 0.00,000,01 and 0.00,000,001 cubic centimeters. The same amount of Type I culture which was fatal to rabbits was fatal to mice. The following table (Table VII) shows the results of tests on the virulence of the Type I culture for rabbits over a period of six months.

TABLE VII.—*Virulence of Type I pneumococcus for rabbits.*

Date.	Dose.	Results.
	Cubic centimeters.	
Feb. 24, 1919	0. 00, 000, 001	+42 hours.
	0. 00, 000, 001	+42 hours.
Mar. 19, 1919	0. 00, 000, 01	+22 hours.
July 9, 1919	0. 00, 000, 01	+17 hours.
	0. 00, 000, 001	+31 hours.
Aug. 21, 1919	0. 00, 000, 01	+42 hours.
	0. 00, 000, 001	+24 hours.
Aug. 23, 1919	0. 00, 000, 01	+37 hours.
	0. 00, 000, 001	+38½ hours.
Aug. 27, 1919	0. 00, 000, 1	+50 hours.
	0. 00, 000, 01	+76 hours.
	0. 00, 000, 001	Survived.

[2] Tropin tests carried out by Miss Alice C. Evans.

Types II and III, on the other hand, were much less virulent for rabbits. A number of attempts were made to determine the virulence of these cultures for rabbits but the results were not satisfactory. In the case of Type II irregularities were usually present. An illustration of this is shown in the following table:

TABLE VIII.—*Virulence of Type II pneumococcus for rabbits and mice.*

Rabbit.	Weight.	Dose.	Results.	Organism.
	Grams.	*Cubic centimeters.*		
1c	3,000	0.1	Survived	
2c	2,500	0.01	+2 days	Pneumococcus isolated.
3c	2,500	0.00,1	+4 days	Pneumococcus not isolated.
4c	2,150	0.00,01	Survived	
5c	1,950	0.00,00,1	+2 days	Pneumococcus isolated.
6c	1,930	0.00,000,1	+2 days	Pneumococcus isolated.
7c	1,850	0.00,000,01	+1 day	Pneumococcus not isolated.
8c		0.00,000,001	Survived	

Mouse.	Dose.	Results.	Organism isolated.
	Cubic centimeter		
1	0.00,01	Chloroformed after 6 hours	Pneumococcus.
2	0.00,001	+23 hours	Pneumococcus.
3	0.00,000,1	+23 hours	Pneumococcus.
4	0.00,000,01	+27 hours	Pneumococcus.
5	0.00,000,001	Survived	

Attempts to stabilize the virulence of the culture by growing in rabbit blood and by passage through rabbits failed.

Type III was only slightly virulent for rabbits. The following results (Table IX) were obtained with the same culture of Type III injected into mice and rabbits.

TABLE IX.—*Virulence of Type III pneumococcus for rabbits and mice.*

	Dose.	Rabbits.	Mice.	Organism isolated.
	Cubic centimeters.			
9c	0.1	+90 hours		Pneumococcus.
10c	0.01	Survived		
11c	0.00,1	Survived		
12c	0.00,01	Survived	+42 hours	Pneumococcus.
13c	0.00,001	Survived	+42 hours	Pneumococcus.
14c	0.00,000,1	Survived	+42 hours	Pneumococcus.
15c	0.00,000,01	Survived	Survived	
16c	0.00,000,001	Survived	Survived	

The amounts of culture fatal to the rabbits also fluctuated somewhat, varying from 0.1 cubic centimeter to 1 cubic centimenter in different tests.

The irregular results shown above indicate the difficulty of obtaining any satisfactory results with cultures which are so variable in virulence. The accompanying protocol (Table X) shows the results with Types II and III Hygienic Laboratory vaccines, the rabbits having been inoculated three times intravenously with vaccine containing 3,000,000,000 organisms per cubic centimeter and the cultures inoculated 13 days after the last inoculation of vaccine.

TABLE X.—*Active immunity in rabbits inoculated intravenously with Type II and III vaccines.*

TYPE II.

	Weight.	Amount of culture.	Result.	Pneumococcus isolated.
Vaccinated rabbits:	*Grams.*	*Cubic centimeters.*		
No. 6d	1,660	0.1	Survived	
No. 5d	1,700	0.01	Survived	
No. 3d	1,840	0.00,1	+45 hours	(+)
No. 2d	1,910	0.00,01	Survived	
No. 4d	2,280	0.00,001	Survived	
No. 1d	2,410	0.00,000,1	Survived	
Control rabbits (no vaccine):				
No. 14d	1,660	0.1	+93 hours	(+)
No. 16d	1,720	0.01	+12 days	(−)
No. 18d	1,920	0.001	Survived	
No. 17d	2,100	0.00,01	Survived	
No. 21d	2,120	0.00,001	Survived	
No. 19d	2,430	0.00,000,1	Survived	

TYPE III.

Vaccinated rabbits:				
No. 12d	1,700	1	+7 days	(−)
No. 7d	1,750	0.5	Survived	
No. 10d	1,825	0.1	Survived	
No. 8d	1,900	0.01	Survived	
No. 9d	1,945	0.001	+76 hours	(+)
No. 11d	1,945	0.00,01	Survived	
Control rabbits (no vaccine):				
No. 22d	1,700	1	Survived	
No. 20d	1,850	0.5	+24 hours	(+)
No. 13d	2,070	0.1	+5 days	(−)
No. 23d	2,100	0.01	Survived	
No. 24d	2,180	0.00,1	Survived	
No. 15d	2,500	0.00,01	Survived	

EXPERIMENTS WITH MICE.

Type I vaccine.—Tests were carried out on mice with the same experimental vaccines which were used on rabbits and which had afforded very high protection. Several series of tests were made, using mice which had been vaccinated with the vaccine 1, 2, and 3 times, the intervals between vaccinations being 4 to 6 days and the culture being given about the same length of time after the last inoculation of vaccine. The results are shown in the accompanying table (Table XI):

TABLE XI.—*Active immunity tests on mice inoculated intraperitoneally with Type I pneumococcus vaccines.*

Amount of culture.	One inoculation.		
	Vaccine A.	Vaccine B.	Controls.
0.00,01 cubic centimeter	+27 hours	+27 hours	+27 hours.
0.00,001 cubic centimeter.	Survived	+2 hours	+19 hours.
0.00,000,1 cubic centimeter.	Survived	Survived	+45 hours.
0.00,000,1 cubic centimeter.	Survived	Survived	+43 hours.
0.00,000,01 cubic centimeter.	+2 hours	Survived	+29 hours.
0.00,000,01 cubic centimeter.	Survived	Survived	+31 hours.
0.00,000,001 cubic centimeter	Survived	Survived	+45 hours.
0.00,000,001 cubic centimeter	Survived	Survived	+43 hours.
	Protection against 1,000 fatal doses.	Probable protection against 1,000 fatal doses.	

TABLE XI.—*Active immunity tests on mice inoculated intraperitoneally with Type I pneumococcus vaccines*—Continued.

Amount of culture.	Two inoculations.		
	Vaccine A.	Vaccine B.	Controls.
0.01 cubic centimeter.	+17 hours	+27 hours	
0.01 cubic centimeter.	Survived	+66 hours	
0.00,1 cubic centimeter.	+27 hours	Survived	
0.00,1 cubic centimeter.	+27 hours	+24 hours	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,001 cubic centimeter.	Survived	Survived	
0.00,001 cubic centimeter.	Survived	Survived	
0.00,000,01 cubic centimeter.			+42 hours.
0.00,000,01 cubic centimeter.			+42 hours
0.00,000,001 cubic centimeter.			+48 hours.
0.00,000,001 cubic centimeter.			+48 hours.
	Protection against 10,000 fatal doses.	Protection against 10,000 fatal doses.	

Amount of culture.	Three inoculations.		
	Vaccine A.	Vaccine B.	Controls.
0.1 cubic centimeter.	+19 hours	+19 hours	
0.1 cubic centimeter.		+21 hours	
0.01 cubic centimeter.	Survived	+22 hours	
0.01 cubic centimeter.		+21 hours	
0.00,1 cubic centimeter.	Survived	+21 hours	
0.00,1 cubic centimeter.		+74 hours	
0.00,01 cubic centimeter.	Survived	Survived	
0.00,01 cubic centimeter.		Survived	
0.00,000,1 cubic centimeter.			+28 hours.
0.00,000,1 cubic centimeter.			+35 hours.
0.00,000,01 cubic centimeter.			+43 hours.
0.00,000,01 cubic centimeter.			+43 hours.
0.00,000,01 cubic centimeter.			Survived.
0.00,000,001 cubic centimeter.			Survived.
0.00,000,001 cubic centimeter.	Protection against 1,000,000 fatal doses.	Protection against 1,000 fatal doses.	

The following summarizes the results:

	One inoculation.	Two inoculations.	Three inoculations.
	Fatal doses.	*Fatal doses.*	*Fatal doses.*
Protection afforded by vaccine A	1,000	10,000	1,000,000
Protection afforded by vaccine B	1,000	10,000	1,000

Tests on mice—Types II and III vaccines.—Several series of mice were inoculated with Type II and III vaccines, inoculations being made 4 or 5 days apart and the culture inoculated about 5 days after the last inoculation of vaccine. The following results (Table XII) were obtained with a Type II vaccine:

TABLE XII.—*Active immunity tests on mice inoculated intraperitoneally with Type II pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.1 cubic centimeter	+20 hours	
0.1 cubic centimeter	+20 hours	
0.01 cubic centimeter	+24 hours	
0.01 cubic centimeter	+24 hours	
0.00,1 cubic centimeter	+(44 hours	
0.00,1 cubic centimeter	+(44 hours	
0.00,01 cubic centimeter	+(44 hours	
0.00,01 cubic centimeter	+(44 hours	
0.00,001 cubic centimeter	+(44 hours	+(44 hours.
0.00,001 cubic centimeter	+(44 hours	+(44 hours.
0.00,000,1 cubic centimeter	+(44 hours	+(44 hours.
0.00,000,1 cubic centimeter	Survived	+(44 hours.
0.00,000,01 cubic centimeter	Survived	+(44 hours.
0.00,000,01 cubic centimeter	Survived	+(44 hours.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.

Protection was afforded against 1 to 10 fatal doses.

A test of another Type II vaccine gave the following results: (Table XIII) the vaccine being injected twice with an interval of 3 days between the injections. The culture was administered 4 days after the last injection of vaccine.

TABLE XIII.—*Active Immunity tests on mice inoculated intraperitoneally with Type II pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.01 cubic centimeter	+32 hours	
0.01 cubic centimeter	+47 hours	
0.00,1 cubic centimeter	+32 hours	
0.00,1 cubic centimeter	+35 hours	
0.00,01 cubic centimeter	+35 hours	
0.00,01 cubic centimeter	+33 hours	
0.00,001 cubic centimeter	+(46 hours	
0.00,001 cubic centimeter	+(46 hours	
0.00,000,1 cubic centimeter	+(46 hours	+32 hours.
0.00,000,1 cubic centimeter	+(46 hours	
0.00,000,01 cubic centimeter	Survived	+35 hours.
0.00,000,01 cubic centimeter	Survived	
0.00,000,001 cubic centimeter	+53 hours	+35 hours.
0.00,000,001 cubic centimeter	+37 hours	
0.00,000,000,1 cubic centimeter	Survived	+37 hours.
0.00,000,000,1 cubic centimeter	Survived	

Some protection was shown in that a control mouse died on the dose of 0.00,000,000,1 cubic centimeter, while the vaccinated mice on 0.00,000,01 cubic centimeter survived.

A test with a Type III vaccine (3 billion organisms per cubic centimeter) showed the following results (Table XIV). The mice were given two inoculations 4 days apart and the culture administered 4 days after the last inoculation.

TABLE XIV.—*Active immunity tests on mice inoculated intraperitoneally with Type III pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.01 cubic centimeter	+35 hours	
0.00,1 cubic centimeter	+29 hours	
0.00,01 cubic centimeter	+27 hours	
0.00,001 cubic centimeter	+27 hours	
0.00,000,1 cubic centimeter	Survived	+37 hours.
0.00,000,01 cubic centimeter	+66 hours	+41 hours.
0.00,000,001 cubic centimeter	+41 hours	+40 hours.
0.00,000,000,1 cubic centimeter	Survived	Survived.

The test shows some protection in that the vaccinated mouse receiving 0.00,000,1 cubic centimeter survived, although the mice on the next two smaller doses died.

Another Type III vaccine also containing 3,000,000,000 organisms per cubic centimeter was tested with the following results (Table XV):

TABLE XV.—*Active immunity tests on mice inoculated intraperitoneally with Type III pneumococcus vaccine (2 inoculations).*

Amount of culture.	Vaccinated mice.	Controls.
0.1 cubic centimeter	+20 hours	
0.1 cubic centimeter	+20 hours	
0.01 cubic centimeter	+26 hours	
0.01 cubic centimeter	+44 hours	
0.00,1 cubic centimeter	+26 hours	
0.00,1 cubic centimeter	+44 hours	
0.00,01 cubic centimeter	+44 hours	
0.00,01 cubic centimeter	+44 hours	
0.00,001 cubic centimeter	+44 hours	+44 hours.
0.00,001 cubic centimeter	+44 hours	+44 hours.
0.00,000,1 cubic centimeter	+44 hours	+44 hours.
0.00,000,1 cubic centimeter	+44 hours	+44 hours.
0.00,000,01 cubic centimeter	+44 hours	+44 hours.
0.00,000,01 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,001 cubic centimeter	Survived	Survived.
0.00,000,000,1 cubic centimeter	Survived	Survived.
0.00,000,000,1 cubic centimeter	Survived	Survived.

In this test apparently no protection was afforded with the Type III vaccine.

The experimental work with Types II and III vaccines on mice indicate that much less protection was afforded by the particular vaccines used than was afforded by the Type I vaccine. The results with Type II are probably better than those with Type III.

The injections of these two vaccines in the doses used (1,500,000,000 organisms at each injection) seemed to act adversely on the mice, as indicated by the roughened fur and appearance of unthrift. That better results could be obtained with a much more dilute vaccine is suggested by the following test (Table XVI). The mice received one inoculation of vaccine and 10 days later cultures were injected. One series of mice received a vaccine containing 100,000,000 organisms per cubic centimeter and the other contained 500,000,000.

TABLE XVI.—*Active immunity tests on mice inoculated intraperitoneally with Type II vaccines diluted.*

VACCINE A (100,000,000 ORGANISMS).

Amount of culture.	Vaccinated mice.	Controls.
0.00,1 cubic centimeter	Survived	0.00,000,1; +22 hours.
0.00,1 cubic centimeter	Survived	0.00,000,1; +36 hours.
0.00,01 cubic centimeter	Survived	0.00,000,01; +46 hours.
0.00,01 cubic centimeter	Survived	0.00,000,01; survived.
0.00,001 cubic centimeter	Survived	0.00,000,001; survived.
0.00,001 cubic centimeter	Survived	0.00,000,001; survived.
0.00,000,1 cubic centimeter	+46 hours (pneumococcus)	
0.00,000,1 cubic centimeter	Survived	
0.00,000,01 cubic centimeter	Survived	
0.00,000,01 cubic centimeter	Survived	

VACCINE B (500,000,000 ORGANISMS).

0.00,1 cubic centimeter	Survived
0.00,1 cubic centimeter	+31 hours (pneumococcus)
0.00,01 cubic centimeter	+22 hours (pneumococcus)
0.00,01 cubic centimeter	+46 hours (pneumococcus)
0.00,001 cubic centimeter	Survived
0.00,001 cubic centimeter	+30 (not pneumococcus)
0.00,000,1 cubic centimeter	+46 (pneumococcus)
0.00,000,1 cubic centimeter	+48 (pneumococcus)
0.00,000,01 cubic centimeter	+46 (pneumococcus)
0.00,000,01 cubic centimeter	+46 (pneumococcus)
0.00,000,001 cubic centimeter	Survived
0.00,000,001 cubic centimeter	Survived

In certain of the tests made on the polyvalent commercial vaccines much higher protection was afforded against Types II and III, particularly Type II, than was found in the tests made with the experimental vaccines injected separately and the results did not seem to bear any definite relation to the concentration of the vaccine. It is possible that there is a certain amount of cross protection, i. e., high protection in the case of Type I may afford some protection against Types II and III.

In accordance with the results of the experimental work carried out it seemed advisable to use mice in making tests on the commercial vaccines. The virulence of the three types can be maintained at fairly definite levels. A larger number of animals can also be employed than if rabbits were used.

In order to limit the tests to a reasonable number of mice it seemed best to arrange the tests somewhat as follows:

Twenty-four [3] vaccinated and twelve control mice to be used in testing a vaccine which contains the three types of pneumococcus. This allows for eight dilutions of each culture for the vaccinated mice, ranging from 0.01 cubic centimeter to 0.00,000,000,1 cubic centimeter and for four dilutions of each culture for the control mice ranging from 0.00,000,1 cubic centimeter to 0.00,000,000,1 cubic centimeter. Two inoculations to be given 4–5 days apart, and the culture dilutions the same length of time after the last inoculation of vaccine.

[3] As a rule 10 or 12 extra mice were vaccinated to allow for deaths before the cultures were inoculated and for repetitions of doubtful tests.

The following protocols (Table XVII–XVIII) illustrate tests put on in this way, the first protocol showing a test on a vaccine which afforded protection against all three types, and the second a test on one which afforded no protection whatever. In stating the amount of protection afforded by a given vaccine it seems necessary to consider survivals which occur in irregular order as well as those occurring in regular order. In the case of the control unvaccinated mice it was found as a rule that the deaths occur in regular order (not necessarily as to number of hours however), i. e., if a mouse died on a certain dilution of the culture all mice on lower dilutions also died. In the case of the vaccinated mice, however, mice on certain of the lower dilutions sometimes survived, while those on the smaller doses succumbed. This occurred so frequently that it seemed to be of some significance. Accordingly these survivals were treated as if they had occurred in order.

The point may be illustrated under Type III in Table XVII. The mouse receiving 0.00,000,01 cubic centimeter which survived is considered in place of the mouse receiving 0.00,000,001 cubic centimeter which died, i. e., the two are reversed in order and the statement may be made that protection was afforded against one fatal dose.

TABLE XVII.—*Test of pneumococcus vaccine.*

[Inoculation of mice: Jan. 7, 0.5 cubic centimeter vaccine intraperitoneally; Jan. 11, 0.5 cubic centimeter vaccine intraperitoneally; Jan. 15, 10 a. m., cultures.]

TYPE I.

Amount of culture.	Vaccinated mice.	Control mice.	Protection against—
0.01 cubic centimeter	+46 hours		
0.00,1 cubic centimeter	Survived		
0.00,01 cubic centimeter	Survived		
0.00,001 cubic centimeter	Survived		
0.00,000,1 cubic centimeter	Survived	+43 hours	1,000,000 fatal doses of Type I.
0.00,000,01 cubic centimeter	+47 hours [a]	+51 hours	
0.00,000,001 cubic centimeter	Survived	+76 hours	
0.00,000,000,1 cubic centimeter	Survived	+37 hours	

TYPE II.

0.01 cubic centimeter	+67 hours		
0.00,1 cubic centimeter	+39 hours		
0.00,01 cubic centimeter	+41 hours		
0.00,001 cubic centimeter	Survived		
0.00,000,1 cubic centimeter	+47 hours	+51 hours	1 fatal dose of Type II.
0.00,000,01 cubic centimeter	+59 hours	+31 hours	
0.00,000,001 cubic centimeter	+71 hours	+56 hours	
0.00,000,000,1 cubic centimeter	Survived	Survived	

TYPE III.

0.01 cubic centimeter	+56 hours		
0.00,1 cubic centimeter	+43 hours		
0.00,01 cubic centimeter	+41 hours		
0.00,001 cubic centimeter	+43 hours		
0.00,000,1 cubic centimeter	+46 hours	+27 hours	
0.00,000,01 cubic centimeter	Survived	+51 hours	1 fatal dose of Type III.
0.00,000,001 cubic centimeter	+56 hours	+51 hours	
0.00,000,000,1 cubic centimeter	Survived	Survived	

[a] Pneumococcus not isolated.

TABLE XVIII.—*Test of pneumococcus vaccine.*

[Inoculations of mice: Oct. 23, 1920, 0.5 cubic centimeter intraperitoneally; Oct. 26, 1920, 0.5 cubic centimeter intraperitoneally; Oct. 30, 1920, 0.5 cubic centimeter cultures.]

TYPE I.

Amount of culture.	Vaccinated mice.	Control mice.	Protection against—
0.01 cubic centimeter	+46 hours		
0.00,1 cubic centimeter	+42 hours		
0.00,01 cubic centimeter	+42 hours		
0.00,001 cubic centimeter	+48 hours		
0.00,000,1 cubic centimeter	+38 hours		
0.00,000,01 cubic centimeter	+48 hours	+34 hours	
0.00,000,001 cubic centimeter	+38 hours	+48 hours	1 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	+60 hours	

TYPE II.

0.01 cubic centimeter	+26 hours		
0.00,1 cubic centimeter	+26 hours		
0.00,01 cubic centimeter	+26 hours		
0.00,001 cubic centimeter	+42 hours		
0.00,000,1 cubic centimeter	+26 hours		
0.00,000,01 cubic centimeter	+34 hours	+26 hours	
0.00,000,001 cubic centimeter	Survived	Survived	0 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	Survived	

TYPE III.

0.01 cubic centimeter	+38 hours		
0.00,1 cubic centimeter	+50 hours		
0.00,01 cubic centimeter	+46 hours		
0.00,001 cubic centimeter	+46 hours		
0.00,000,1 cubic centimeter	+34 hours		
0.00,000,01 cubic centimeter	+48 hours	+42 hours	
0.00,000,001 cubic centimeter	Survived	Survived	0 fatal dose.
0.00,000,000,1 cubic centimeter	Survived	Survived	

The results obtained in testing other commercial vaccines according to this method are shown in tabular form at the end of the article.

DISCUSSION.

The results of the tests carried out on the commercial vaccines as well as the experimental work on the vaccines of known composition show that the amount of protection afforded to mice may be measured rather definitely and comparison made as to the relative value of the vaccines in question. It would be reasonable to assume that a vaccine which gave entirely negative results in the test used would not be of value as a prophylactic agent. The indication would be that there was something faulty in the method of preparation of the vaccine. If protection were afforded against 100,000 to 1,000,000 or even a smaller number of fatal doses of culture the indications would be that such a vaccine would be of some definite value.

In general the vaccines tested produced considerable immunity against Type I pneumococcus (with the exception of several which showed no protection whatever against any of the types). As in the case of pneumococcus serum, so in pneumococcus vaccine most satisfactory results are to be expected with Type I. Only slight

protection was afforded by the vaccines against Type III pneumococcus. Type II appeared to occupy an intermediate position in relation to the other two.

Regarding the amounts of vaccine suitable for testing on mice it was thought that the most information could be obtained by using in the preliminary work uniform amounts of vaccine as to number of organisms and using the same amounts as to volume in testing the commercial vaccines. In the preliminary experimental work vaccines containing 3 billion organisms per cubic centimeter were used for the most part. The mice uniformly received 0.5 cubic centimeter of vaccine intraperitoneally at each inoculation and the rabbits either 0.5 or 1 cubic centimeter for the first and 1 cubic centimeter for the second and third inoculations. A series of tests was carried out on mice in which the Hygienic Laboratory vaccines were diluted, the mice being inoculated with vaccines containing 50,000,000, 100,000,000, 250,000,000, 500,000,000, 1,000,000,000, and 1,500,000,000 organisms. A commercial vaccine containing 10,000,000,000 organisms was diluted to 2,500,000,000 and 500,000,000 organisms, and tests carried out with the original and with the diluted vaccines. The commercial vaccines tested ranged from 100,000,000 to 20,000,000,000 organisms per cubic centimeter. On comparing the results obtained in all of these tests it appears that no definite statements can be made as to what concentration of organisms is most favorable for affording protection in mice. It seems that a vaccine which is decidedly dilute may afford very good protection. Good results were also obtained with very concentrated vaccines though at times it appeared that the doses used may have been overwhelming and what may be likened to a negative phase brought out. This seemed to be particularly true of Types II and III vaccines.

The matter of testing pneumococcus vaccines for their probable value for man would seem therefore to be a question of determining either on man or monkeys the concentration and dose of vaccine which is most likely to be of value and then so adapting the test to mice as best to show this value. This would probably involve diluting the vaccine to a certain point before injecting into mice. A dose which is suitable for man seems quite likely to be an overwhelming one for a mouse.

As to the length of immunity conferred as the result of the use of pneumococcus vaccine some information was obtained incidental to this work as far as animals are concerned. Tests were carried out at intervals on the surviving rabbits in the series of 18 rabbits referred to in the first table and also on another series of rabbits. It was found that six months after the administration of the vaccine protection was afforded against 100 fatal doses, as against 100,000 fatal doses 16 days after the last inoculation of vaccine. After 9 months there was practically no protection.

SPECIFICITY.

The possible cross protection between the different types has been referred to. A test was also carried out with a staphylococcus vaccine to determine the specificity of pneumococcus vaccines as regards other organisms. A series of mice was inoculated with this vaccine in the same way as with the pneumococcus vaccine. No protection whatever was afforded against Types I, II, or III pneumococcus cultures.

In conclusion it may be stated, considering the results as a whole, that apparently the potency testing of pneumococcus vaccine can be carried out with reasonably satisfactory results. Though irregularities are to be expected, the experiments reported show some decidedly definite and clear-cut results. The test for active immunity is undoubtedly the most effective test and the superiority of mice over rabbits has been shown.

It is evident that the protective property afforded as the result of vaccination resides in the serum only to a limited extent and although it can not be demonstrated very satisfactorily through the presence of agglutinins, tropins, precipitins, or bacteriostatic properties, it may nevertheless exist and can be shown by inoculating the vaccinated animal itself with the live culture. This test is decisive, while in carrying out tests for determining the presence of the antibodies mentioned the impression is gained that although these tests may give some information, they are too uncertain to be of great practical value. The same may be said in general of tests for determining passive immunity by protection tests on other animals than those receiving the vaccine. In work previously reported by the writer in testing the comparative value of saline and oil vaccines the mouse protection test was made use of to some extent and the results while giving some information were not very striking. Mouse protection tests with serum from inoculated persons were also used by Cecil and his coworkers in their early work and a certain number of positive results were obtained. The recent publications of Cecil and associates show, however, that in monkeys there may be complete immunity against pneumonia, though protective antibodies are very often absent in the serum.

SUMMARY.

Tests were carried out on rabbits and mice to determine their suitability for testing the potency of pneumococcus vaccine.

Agglutination, precipitin and tropin tests were made on the serum and tests designed to show bacteriostatic action were made on the whole blood of vaccinated rabbits. Tests for determining active immunity in rabbits using Type I pneumococcus vaccine were also carried out. The tests for active immunity as shown by direct

inoculation of the live cultures in the vaccinated animals were by far the most conclusive. Tests on the serum and whole blood were suggestive but not sufficiently definite for practical use.

Tests for active immunity in rabbits produced by Types II and III vaccine were inconclusive owing to the fluctuating or low virulence of these types for rabbits.

Tests for active immunity produced in mice were found to be most satisfactory for the testing of polyvalent pneumococcus vaccines containing Types I, II, and III. The superiority of mice over rabbits was due to the fact that the virulence of the three fixed types can be maintained at a very uniform level for this species.

In testing Types I, II, and III experimental vaccines separately in mice, the best results were obtained with Type I vaccine. Only a small amount of protection was afforded against Type III. Decidedly less protection was afforded by Type II than by Type I vaccine. In the testing of polyvalent vaccines, more definite protection was afforded against Types II and III, suggesting the possibility of a certain amount of cross protection.

Immunity in rabbits against Type I pneumococcus lasted to a moderate extent for 6 months and disappeared in 9 months.

Commercial polyvalent pneumococcus vaccines afforded protection in mice against doses of culture ranging from 0 to 1,000,000 fatal doses in the case of Type I, 0 to 10,000 fatal doses in the case of Type II and 0 to 100 fatal doses in the case of Type III.

Tests on commercial pneumococcus vaccines.

Vaccine.	Dose.	Number of inoculations.	Total dosage.	Dates of inoculations of vaccine.	Date of inoculation of culture.	Protection afforded (fatal doses).		
						Type I.	Type II.	Type III.
	Cubic centimeter.			1919.	1919.			
A	0.5	2	1 cubic centimeter (750,000,000 organisms).	Nov. 14, 21	Nov. 29	1,000	10	10
				1920.	1920.			
B	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Mar. 19, 23	Mar. 27	1,000	10	1
C	0.5	2	1 cubic centimeter (400,000,000 organisms.)	June 3, 7	June 12	1	10,000	0
D	0.5	2	1 cubic centimeter	July 9, 13	July 17	1,000,000	100	10
E	0.5	2	1 cubic centimeter (10,000,000,000 organisms).	July 22, 26	July 31	1,000,000	1–10	0
F	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Aug. 10, 14	Aug. 18	100	10,000	10
G	0.5	2	1 cubic centimeter (20,000,000,000 organisms).	Oct. 23, 26	Oct. 30	1	0	0
H	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Nov. 11, 15	Nov. 20	10–100	1,000	1
I	0.5	2	1 cubic centimeter (9,000,000,000 organisms).	Nov. 11, 15	Nov. 20	100	1–10	0

Tests on commercial pneumococcus vaccines—Continued.

Vaccine.	Dose.	Number of inoculations.	Total dosage.	Dates of inoculations of vaccine.	Date of inoculation of culture.	Protection afforded (fatal doses).		
						Type I.	Type II.	Type III.
	Cubic centimeter.			1920.	1920.			
K	0.5	2	1 cubic centimeter	Dec. 3, 7	Dec. 12	100	0	10
L	0.5	2	1 cubic centimeter (4,000,000,000 organisms).	Dec. 3, 7	Dec. 12	1,000	1,000	1,000
M	0.5	2	1 cubic centimeter (20,000,000,000 organisms).	Dec. 14, 22	Dec. 28	100	100	1
N	0.5	2	1 cubic centimeter (500,000,000 organisms).	Dec. 14, 24	Dec. 28	1,000	1,000	0
O	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Dec. 30 1921. Jan. 4	1921. Jan. 8	0	0	0
P	0.5	2	1 cubic centimeter (100,000,000 organisms).	1920. Dec. 31 1921. Jan. 4	Jan. 8	10	100	1
Q	0.5	2	1 cubic centimeter	Jan. 7, 11	Jan. 15	1,000,000	1	1
R	0.5	2	1 cubic centimeter (500,000,000 organism).	Jan. 7, 11	Jan. 15	10,000	10,000	1
S	0.5	2	1 cubic centimeter (1,000,000,000 organisms)	Feb. 11, 15	Feb. 10	0	0	0
T	0.5	2	1 cubic centimeter (1,000,000,000 organisms).	Feb. 18, 24	Mar. 1	0	0	0
U	0.5	2	1 cubic centimeter (3,000,000,000 organisms).	Mar. 5, 9	Mar. 12	10	100	100
V	0.5	2	1 cubic centimeter (4,000,000,000 organisms).	Mar. 5, 9	Mar. 12	1,000	10,000	100

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III. THE ADAPTABILITY OF VARIOUS AMERICAN PEPTONES FOR USE IN CHOLERA MEDIA.[1]

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INTRODUCTION.

The work here reported was undertaken for the purpose of ascertaining whether the various American peptones can be used as effectively as Witte's peptone, called for in Goldberger's [2] formulæ for cholera media. The opportunity was also afforded in this connection to study in terms of H ion concentration the reaction most favorable for the development of *V. choleræ*.

The advantage of the Goldberger media lies in the simplicity and ease of their preparation and the availability of the ingredients. The particular advantage lies in the fact that Goldberger found it unnecessary to adjust the reaction except empirically. This may be explained by the following considerations: Approximate uniformity in reaction of Witte's peptone; the use of beef extract (Liebig's) and not meat infusion in the media; the fact that the cholera vibrio develops over a considerable range of reaction and that alkaline egg mixtures inhibit *B. coli*.

Dunham's solution unadjusted in reaction is usually recommended as a favorable enrichment medium to be used prior to streaking on plates for isolation. Many cholera cultures probably have a natural tendency to overgrow *B. coli* in Dunham's solution, particularly if they are numerous. Here the question of reaction probably does not enter in greatly, unless the peptone is one which produces a rather acid medium. If, on the other hand, the strain is one which does not have a tendency to multiply rapidly or is present in small numbers, it is necessary to make conditions more favorable for the growth of the cholera organism by rendering them less favorable for *B. coli*. Advantage is taken of the fact that *V. choleræ* is able to develop at a reaction which is more alkaline than is tolerated by *B. coli*, and it is in this connection that the alkaline egg enrichment medium of Goldberger is of particular value.

This paper is mainly concerned with the two enrichment media, which Goldberger recommends be used conjointly, and the alkaline egg agar plating medium adapted from a similar medium described earlier by Krumwiede, Prátt and Grund.[3]

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[1] Manuscript submitted for publication Oct. 4, 1923.

[2] Hygienic Laboratory Bulletin, 1914, No. 91, pp. 19–39.

[3] Jour. Infect. Dis., 1912, 10, 134–141.

EXPERIMENTAL WORK.

The peptone enrichment solution of Goldberger, which is modified Dunham's solution, is prepared as follows :

Potassium nitrate	1 gram.
Sodium carbonate (crystallized)	2 grams.
Sodium chloride	100 grams.
Peptone (Witte)	100 grams.
Distilled H_2O	1,000 cubic centimeters.

Dissolve by heating, then filter and distribute in flasks in 100 cubic centimeter quantities; sterilize and store. For use this stock solution is diluted by the addition of 9 volumes of distilled water and this solution distributed in tubes and flasks in quantities of 10 and 50 cubic centimeters, respectively, and sterilized.

The alkaline egg peptone enrichment solution is made as follows:

(*a*) Prepare an alkaline egg solution by first shaking up or beating up an egg with an equal volume of water and then adding to this egg water an equal volume of a 5 per cent solution of anhydrous sodium carbonate. Steam ¾ to 1 hour. (*b*) Prepare Dunham's solution: Peptone 10 grams, salt 5 grams, water 1,000 cubic centimeters. For use mix (*a*) and (*b*) in proportion of 1 to 9.

The alkaline egg agar medium for plating is prepared as follows:

1. Mix thoroughly equal parts of whole egg and water. Filter through thin layer of cotton.

2. Add to No. 1 an equal volume of 6.5 per cent solution of sodium carbonate (anhydrous) and mix thoroughly. Steam in Arnold sterilizer 20 minutes.

3. Prepare an agar as follows: Meat extract (Liebig's) 3, peptone (Witte) 10, sodium chloride (chemically pure) 5, glucose 1, agar 30, distilled (or good quality tap) water 1,000.

Mix 1 part of No. 2 with 5 of boiling hot No. 3 and pour the plates. Allow plates to dry with covers off.

The peptone enrichment solution made according to the above formula contains only 0.0074 per cent of Na_2CO_3 and can not be considered a very alkaline medium. The egg enrichment medium contains 0.25 per cent of Na_2CO_3 and the alkaline egg agar 0.54 per cent. The alkaline egg agar medium of Krumwiede, Pratt, and Grund contains from 0.67 to 0.75 per cent Na_2CO_3, which, however, was found by Goldberger to be too alkaline for the growth of the cholera strains which he studied.

In this paper the formulæ of Goldberger were followed and variations in reactions effected by the use of added normal sodium hydroxide or normal hydrochloric acid.

The strains used include three laboratory strains, No. 87 which is the Naples strain 159, used by Goldberger; No. 95, which is the Austria 13 strain, received from the New York City health department; and No. 93, which was received from the American Museum of Natural History and previously obtained by them from the New York City health department. Two other strains used by Goldberger, No. 88 (Naples 202 strain) and No. 97 (New York 1132)

were also used in part of the work. Later 3 strains recently isolated from cases of cholera in the Philippine Islands and 6 strains recently isolated in the cholera epidemic in Poland were added. In addition to these 1 culture of *B. coli*, Hygienic Laboratory strain 119, 2 cultures of *B. coli* recently isolated from stools, 1 culture of *B. alkaligenes*, Hygienic Laboratory strain 116, and a culture of *B. pyocyaneus*, Hygienic Laboratory strain 279, were used.

The peptones used included the five American peptones Parke, Davis & Co.'s, Fairchild's, Armour's, Difco, and Squibb's, with Witte's as control. As a rule the American peptones produce more acid media than Witte's. This was found to be true of all except Squibb's.[4]

Though there are other factors to be considered the main part of the work resolved itself into ascertaining to what extent differences in reaction of the various peptones used affect the results and to what extent it is necessary or desirable to modify the reactions of the various media. A special study was made of the growth of *V. choleræ* and *B. coli* in the alkaline range above pH 8.

The general plan of the work may be summarized in the following paragraphs:

1. A study was made of the growth curves of *V. choleræ*, *B. coli*, *B. pyocyaneus*, and *B. alkaligenes*, after cultivation in Witte's peptone alkaline egg enrichment medium adjusted to varying reactions.

2. Having determined the favorable reaction for the growth of *V. choleræ* and the suppression of *B. coli* in Witte's alkaline egg peptone medium, tests were carried out to find whether it was desirable to adjust media made with the various American peptones to a corresponding reaction.

3. Tests similar to those described in the preceding paragraph, were made with mixtures of *V. choleræ* and *B. coli*.

4. Determination of the reaction of Witte's alkaline egg agar plating medium suitable for the growth of *V. choleræ* and inhibitory to *B. coli*.

5. Growth of *V. choleræ* and *B. coli* on alkaline egg agar plates, using the various peptones in two series, the agar being unadjusted in one series, and in the other adjusted to the reaction which had been found favorable in the tests carried out with Witte's peptone.

6. Isolation of the cholera organism. Having obtained information from the preceding tests as to conditions favorable for the growth of *V. choleræ*, the methods were put into practice for isolating the organism from artificially contaminated stools.

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[4] The following peptones were used: Witte's pre-war peptone, not numbered; Squibb's 1E06234; Fairchild's, 170620; Difco, not numbered; Armour's, 9-8-17; Parke, Davis & Co.'s, 231795 and 2486609. (The results for cultures 400 and 404 in Table II were obtained with Parke, Davis & Co.'s peptone No. 2522835.)

1. Growth curves of *V. choleræ*, *B. coli*, *B. alkaligenes*, and *B. pyocyaneus* in Witte's alkaline egg enrichment medium:

The alkaline egg enrichment medium was selected as a medium for determining the growth curves of the various organisms for the reason that it was found to be fairly stable in pH value in the alkaline range. Peptone solutions adjusted to reactions more alkaline than pH 8 were found to be very unstable, the reaction always tending in the direction of neutrality, the more so as the degree of alkalinity was increased. This was observable on sterilization, on standing at room or incubator temperature, and on inoculation with cultures.

It was felt after some work had been done on the subject that the determination of suitable buffer agents to be added to the peptone solution to overcome this difficulty was scarcely justifiable in view of the fact that the alkaline egg medium apparently provided the buffer components necessary to maintain the desired reaction within reasonable limits without the addition of other substances. Though not as stable as might be desired in the more acid reactions used the changes were much less in proportion than in the peptone solutions. Reactions between pH 8.5 and 10 were found to be fairly stable.

The pH value of alkaline egg enrichment medium made with Witte's peptone was about pH 9.5–9.6. Different portions of this medium were adjusted to reactions of pH 8.0, 8.5, 9.0, and 10 by the addition of N/NaOH and N/HCl. The following table and diagrams indicate the results obtained on planting with 0.001 cubic centimeter of the various cultures, incubating for seven hours and plating on glucose agar. (Table I; Diagrams I–II.)

TABLE I.—*Growth of V. choleræ and B. coli in Witte's alkaline egg peptone enrichment media.*

Original pH.	*V. choleræ* 87.			*V. choleræ* 88.			*V. choleræ* 97.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	15,600,000	7.19	8.6	16,800,000	7.23	8.6	8,850,000	6.95	8.6
8.5	980,000	5.99	8.8	2,700,000	6.43	8.8	465,000	5.67	8.6
9	1,710,000	6.23	9.0	1,000,000	6.0	9.0	1,820,000	6.26	8.8
9.5	112,000	5.05	9.4	263,000	5.42	9.2	285,000	5.45	9.2
10	0	------	9.7	0	------	9.6	84,000	4.92	9.7

Original pH.	*V. choleræ* 93.			*V. choleræ* 95.			*B. coli* 119.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	100	2	8.6	2,630,000	6.42	8.0	9,250	3.97	8.6
8.5	23,500	4.37	8.6	16,900,000	7.23	8.0	5,100	3.71	8.6
9	42,000	4.62	9.4	38,800,000	7.59	8.4	1,200	3.08	9.3
9.5	91,500	4.96	9.4	14,100,000	7.15	9.2	200	2.3	9.4
10	300	2.48	10.0	445,000	5.65	10	0	------	9.7

TABLE I.—*Growth of V. choleræ and B. coli in Witte's alkaline egg peptone enrichment media*—Continued.

RECENTLY ISOLATED STRAINS.

Original pH.	*V. choleræ* 409.			*V. choleræ* 400.			*V. choleræ* 399.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	6,250,000	6.8	8.6	4,670,000	6.67	8.6	54,000,000	7.73	8.0
8.5	2,820,000	6.45	8.9	15,400,000	7.19	8.7	31,200,000	7.49	8.4
9	2,290,000	6.36	9.0	5,600,000	6.75	9.0	17,900,000	7.25	8.9
9.5	1,740,000	6.24	9.4	6,900,000	6.84	9.3	437,000	5.64	9.3
10	740	2.87	9.5	420	2.62	9.4	35,000	4.54	9.6

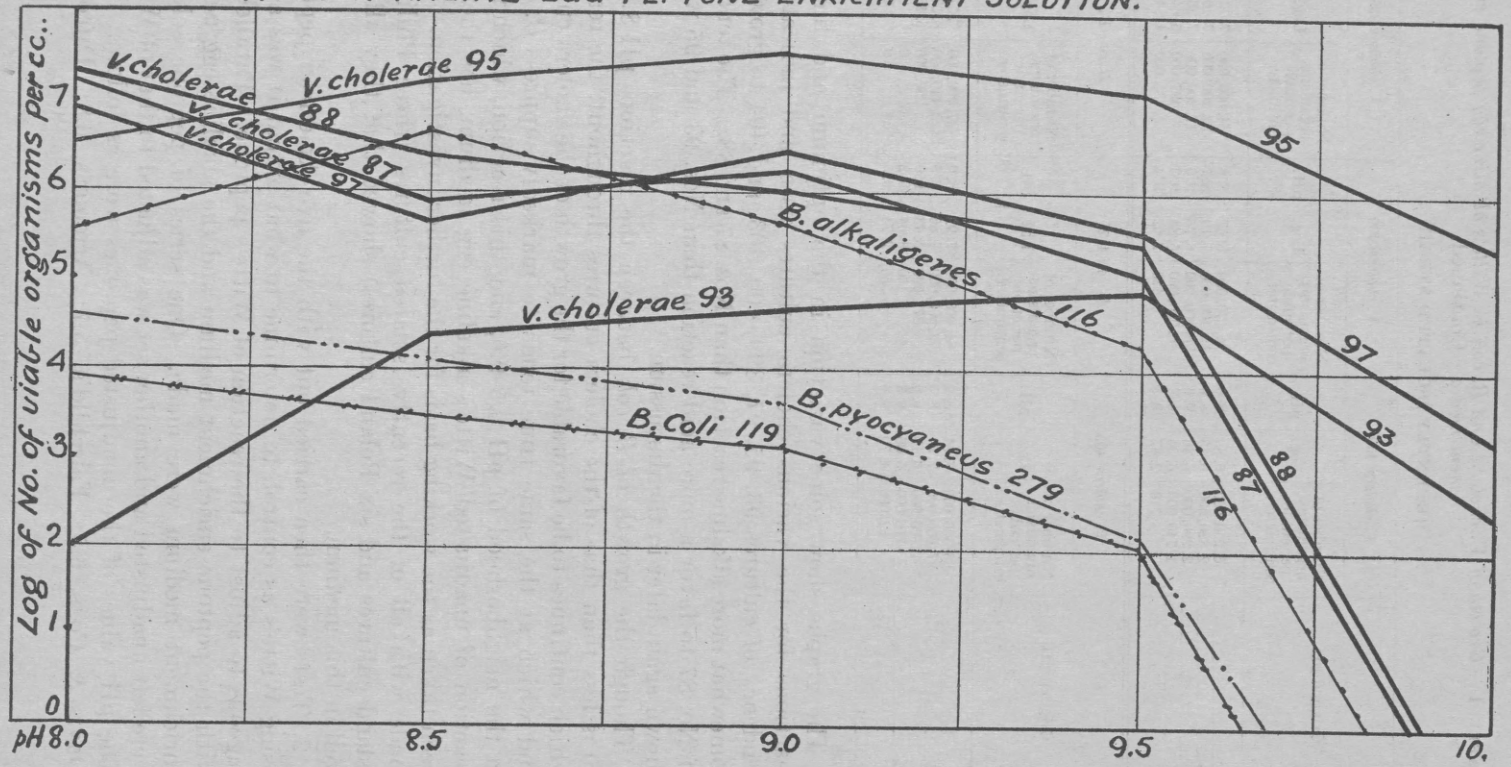
Original pH.	*V. choleræ* 405.			*B. coli* 412.			*B. coli* B.		
	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.	Number of organisms per cubic centimeter.	Log.	pH.
8	86,300,000	7.94	8.2	84,000,000	7.92	8.0	37,000,000	7.57	8.8
8.5	87,000,000	7.94	8.5	72,000,000	7.86	8.5	3,410,000	6.53	8.9
9	1,560,000	6.19	9.0	271,000	5.43	9.0	280,000	5.45	9.2
9.5	116,000	5.06	9.3	1,100	3.04	9.2	0	0	9.3
10	62,000	4.79	9.6	550	2.74	9.5	0	0	9.4

The graphs show some variation in the optimum and limiting reactions for the various cholera cultures used and indicate the tendency of cultures 93, 95, 97, 399, 400, 403, and 404 to grow at a somewhat more alkaline reaction than Nos. 87 and 88. The tendency of No. 87 to favor a more acid reaction than Nos. 93 and 95 will be shown again later in the discussion.

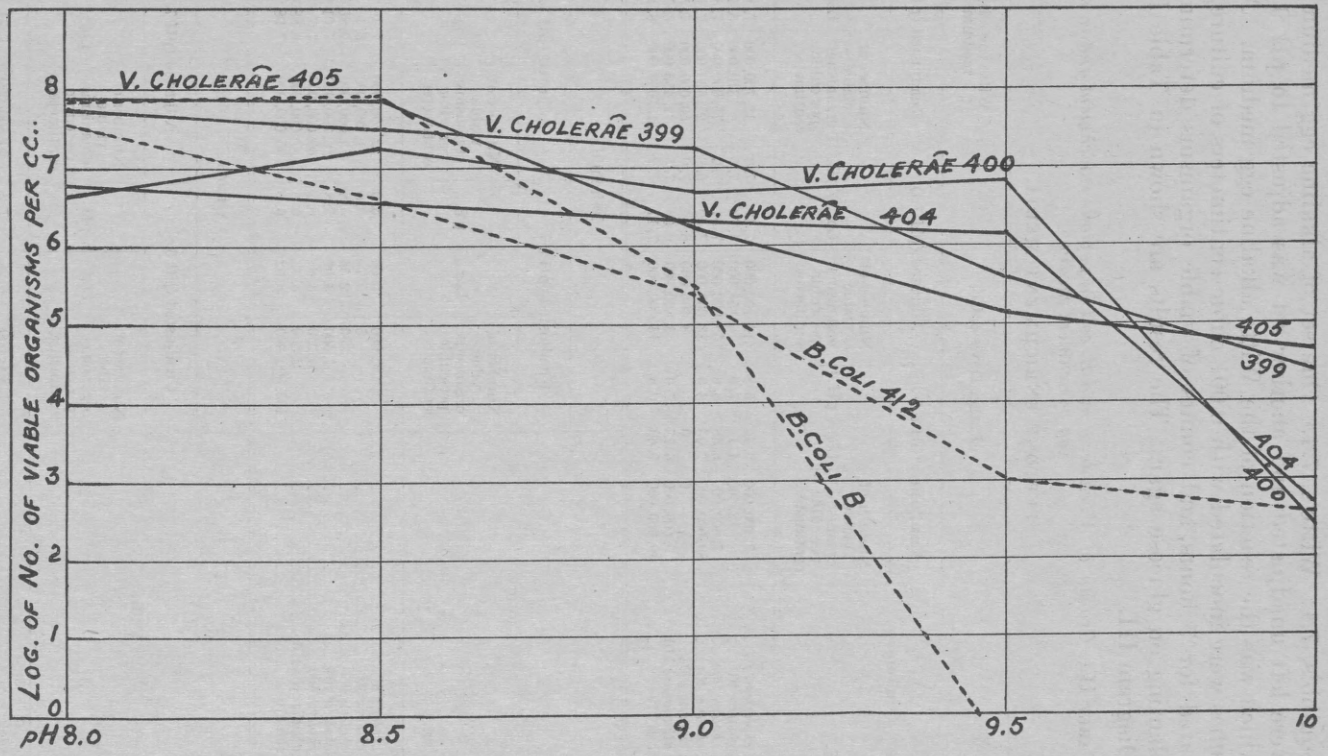
Though the growth of *B. coli* between the reactions pH 8.5 and 10 is less than that of the cholera cultures throughout, the reaction which continues to be favorable for the growth of the cholera cultures and which at the same time tends to markedly suppress *B. coli* is in the neighborhood of pH 9.5–9.6, and this reaction, which is the reaction of unadjusted Witte's alkaline egg medium, has therefore been taken as the working basis for the further investigation. Tests made with all of the recently isolated cultures (three Philippine Island cultures and six Poland cultures) showed that they all grew well in this medium.

2. Tests were then carried out with the five American peptones, using Witte's as control, to determine to what extent it was advantageous to adjust to the reaction of Witte's peptone. Parallel tests with the peptone enrichment medium and the alkaline egg peptone enrichment medium were made. One series of peptone solutions were left unadjusted and another set was adjusted to that of Witte's. The pH values of the unadjusted peptones were as follows: Parke, Davis & Co.'s, 6.5; Fairchild's, 5.7; Armour's, 6.8; Difco, 7.0;

DIAGR.I:- GROWTH OF V. CHOLERAE, B. COLI, B. ALKALIGENES AND B. PYOCYANEUS IN WITTE'S ALKALINE EGG PEPTONE ENRICHMENT SOLUTION.



DIAGR. II :- GROWTH OF V. CHOLERÆ AND B. COLI (RECENTLY ISOLATED CULTURES) IN WITTE'S ALKALINE EGG PEPTONE ENRICHMENT SOLUTION.



Squibb's, 7.4; Witte's, 7.4. One set of alkaline egg medium tubes were left unadjusted and another set was adjusted to pH 9.5–9.6, which was the reaction of the Witte alkaline egg medium. All the tubes were inoculated with 0.001 cubic centimeters of culture, incubated for 7 hours, and counts of viable organisms determined by planting on glucose agar. The results are shown in Table II and Diagram III.

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media.*

PEPTONE ENRICHMENT MEDIA.

Culture.	Parke, Davis & Co.						Witte (no adjusted medium).		
	Unadjusted (pH 6.5).			Adjusted (pH 7.4).			Unadjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.[a]	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	3, 100, 000	6. 49	6. 8	14, 700, 000	7. 17	7. 3	12, 700, 000	7. 1	7. 4
V. choleræ 93	12, 500	4. 1	7. 0	54, 000	4. 73	7. 5	310, 000	5. 49	7. 1
V. choleræ 95	7, 600, 000	6. 88	6. 6	65, 000, 000	7. 81	7. 0	75, 000, 000	7. 88	7. 2
V. choleræ 404	89, 000, 000	7. 95	6. 0	33, 000, 000	7. 52	6. 8	25, 600, 000	7. 41	7. 3
V. choleræ 400	0	0	------	8, 400, 000	6. 92	7. 0	50, 000, 000	7. 7	7. 6
B. alkaligenes 116	5, 600, 000	6. 75	7. 0	3, 800, 000	6. 58	7. 5	2, 450, 000	6. 39	7. 2
B. coli 119	49, 000, 000	7. 69	6. 8	13, 000, 000	7. 11	7. 2	115, 000, 000	8. 1	7. 2

Culture.	Fairchild.					
	Unadjusted (pH 5.7).			Adjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH
V. choleræ 87	1, 150, 000	6. 06	6. 0	12, 500, 000	7. 1	7. 6
V. choleræ 93	5	0. 7	5. 8	1, 260, 000	6. 1	7. 2
V. choleræ 95	350	2. 54	5. 8	73, 000, 000	7. 86	7. 5
V. choleræ 404	4, 600	3. 66	5. 8	10, 300, 000	7. 01	7. 1
V. choleræ 400	6, 250	3. 8	6. 2	50, 000, 000	7. 7	7. 2
B. alkaligenes 116	2, 500	3. 4	5. 8	7, 600, 000	6. 88	7. 5
B. coli 119	105, 000, 000	8. 02	6. 0	95, 000, 000	7. 98	7. 3

Culture.	Armour.					
	Unadjusted (pH 6.8).			Adjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	1, 890, 000	6. 28	7. 2	20, 500, 000	7. 31	7. 5
V. choleræ 93	40, 000	4. 6	6. 8	5, 450, 000	6. 74	7. 2
V. choleræ 95	5, 150, 000	6. 71	6. 8	27, 200, 000	7. 43	7. 3
V. choleræ 404	10, 500, 000	7. 02	6. 8	35, 000, 000	7. 54	7. 4
V. choleræ 400	5, 150, 000	6. 71	6. 8	15, 900, 000	7. 2	7. 2
B. alkaligenes 116	450, 000	5. 65	6. 8	1, 020, 000	6. 01	7. 4
B. coli 119	5, 070, 000	6. 71	6. 8	25, 900, 000	7. 41	7. 4

[a] After incubation.

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media*—Continued.

PEPTONE ENRICHMENT MEDIA—Continued.

Culture.	Difco.						Squibbs (no adjusted media).		
	Unadjusted (pH 7.0).			Adjusted (pH 7.4).			Unadjusted (pH 7.4).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	725,000	5.86	7.6	38,000	5.58	7.8	15,800,000	7.2	7.6
V. choleræ 93	39,000	4.59	7.0	2,200	3.34	7.3	22,500	4.35	7.2
V. choleræ 95	3,100,000	6.49	7.5	16,200,000	6.67	7.8	660,000	5.82	7.4
V. choleræ 404	14,100,000	7.15	7.4	------	------	------	87,000,000	7.94	7.0
V. choleræ 400	38,000,000	7.58	7.4	------	------	------	16,100,000	7.21	7.0
B. alkaligenes 116	21,000	4.32	7.0	380,000	5.58	7.5	53,000	4.72	7.5
B. coli 119	8,400,000	6.92	7.5	11,300,000	7.05	7.6	23,100,000	7.36	7.2

ALKALINE EGG ENRICHMENT MEDIA.

Culture.	Parke, Davis & Co.						Witte (no adjusted media).		
	Unadjusted (pH 9.4).			Adjusted (pH 9.6).			Unadjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	30,000	4.48	9.0	⟨10,000	⟨4	9.2	8,250,000	6.92	9.2
V. choleræ 93	90,000	4.95	9.2	170,000	5.23	9.4	33,900	4.53	9.4
V. choleræ 95	2,800,000	6.45	9.0	9,300,000	6.97	9.2	65,000,000	7.81	9.3
V. choleræ 404	26,000,000	7.42	8.8	14,500,000	7.16	8.9	14,600,000	7.16	9.2
V. choleræ 400	11,300,000	7.05	8.9	7,100,000	6.85	8.9	11,700,000	7.07	9.2
B. alkaligenes 116	39,000	4.59	9.3	1,050	3.02	9.4	81,000	4.99	9.6
B. coli 119	⟨100	⟨2	9.3	⟨100	⟨2	9.4	⟨100	⟨2	9.6

Culture.	Fairchild.					
	Unadjusted (pH 8.9).			Adjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	19,600,000	7.29	8.4	28,000,000	7.45	8.7
V. choleræ 93	1,400	3.15	8.6	2,600	3.45	9.0
V choleræ 95	20,800,000	7.32	8.2	54,000,000	7.73	8.7
V. choleræ 404	10,800,000	7.03	8.8	3,900,000	6.59	9.2
V. choleræ 400	51,000,000	7.71	8.8	2,230,000	6.35	9.2
B. alkaligenes 116	183,000	5.26	8.6	20,500	4.31	8.8
B. coli 119	73,000	4.86	8.4	850	2.93	9.0

TABLE II.—*Growth of V. choleræ and B. coli in peptone enrichment and in alkaline egg enrichment media*—Continued.

ALKALINE EGG ENRICHMENT MEDIA—Continued.

Culture.	Armour.					
	Unadjusted (pH 9.2).			Adjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.
V. choleræ 87	15,500,000	7.19	8.8	35,000,000	7.54	9.2
V. choleræ 93	77,000	4.89	9.0	500	2.7	9.3
V. choleræ 95	4,550,000	6.66	8.8	33,000,000	7.52	9.2
V. choleræ 404	17,200,000	7.24	8.9	25,000,000	7.4	9.2
V. choleræ 400	7,300,000	6.86	8.9	4,450,000	6.65	9.2
B. alkaligenes 116	470,000	5.67	9.0	67,000	4.83	9.3
B. coli 119	2,900	3.46	9.0	⟨100	⟨2	9.3

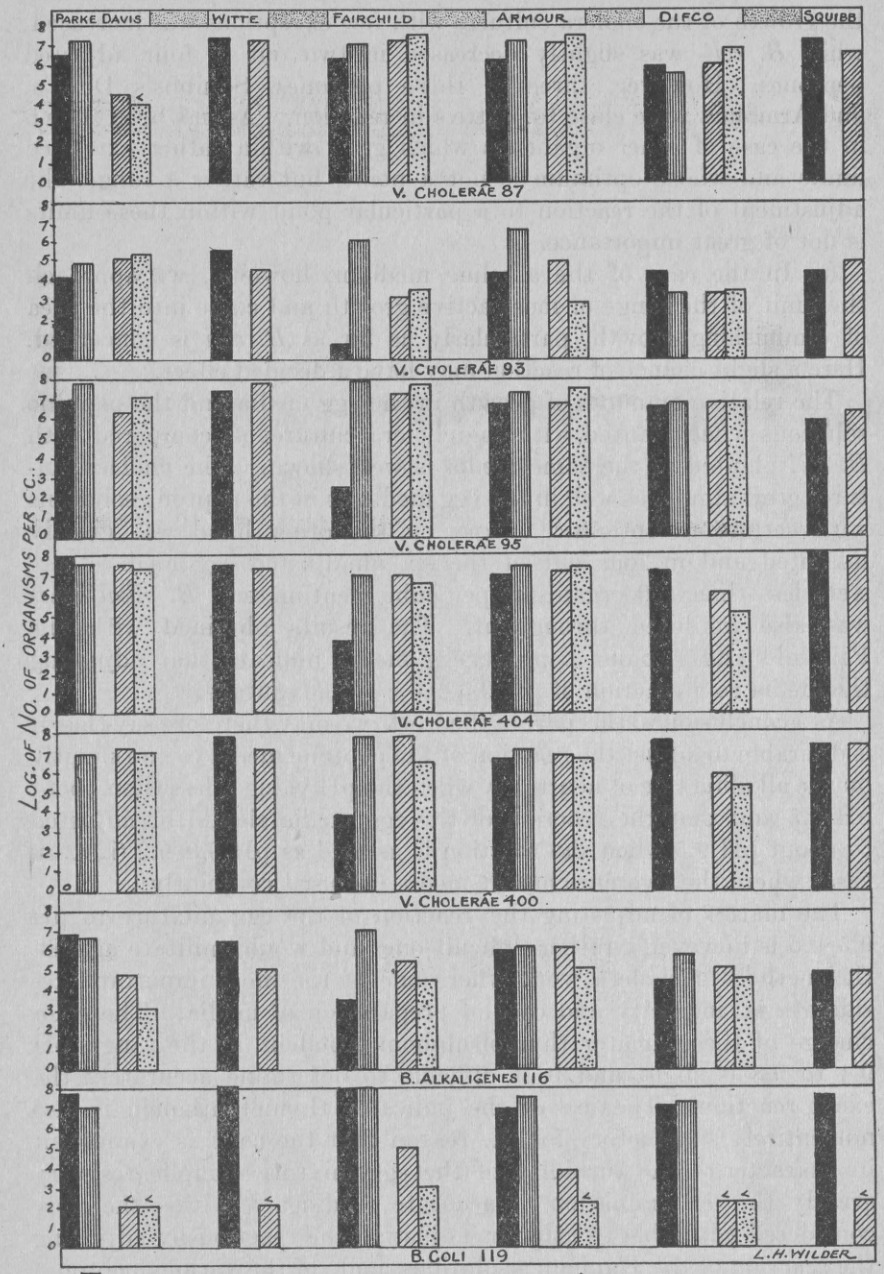
Culture.	Difco.						Squibbs (no adjusted media).		
	Unadjusted (pH 9.3).			Adjusted (pH 9.6).			Unadjusted (pH 9.6).		
	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH.	Number of viable organisms per cubic centimeter.	Log.	pH
V. choleræ 87	1,080,000	6.03	8.6	4,450,000	6.65	9.2	3,730,000	6.57	9.2
V. choleræ 93	1,200	3.3	9.2	2,900	3.46	9.4	56,000	4.75	9.3
V. choleræ 95	5,200,000	6.72	8.7	5,100,000	6.71	9.0	1,850,000	6.27	9.2
V. choleræ 404	1,410,000	6.15	9.3	106,000	5.1	9.5	52,600,000	7.72	9.2
V. choleræ 400	545,000	5.74	9.3	108,000	5.1	9.5	13,500,000	7.13	9.2
B. alkaligenes 116	117,000	5.07	9.2	19,600	4.29	9.4	45,500	4.66	9.4
B. coli 119	⟨100	⟨2	9.2	⟨100	⟨2	9.3	⟨100	⟨2	9.5

NOTE 1.—Parke, Davis & Co.'s peptone used for *V. choleræ* 400 and 404 had a reaction of pH 5.6.
NOTE 2.—The reaction of the alkaline egg medium in the case of cultures 400 and 404 was adjusted by adjusting the reaction of the Dunham's solution before the addition of the egg mixture.

The deductions to be drawn from the table and diagram are:

(*a*) That all the cholera cultures grew well in unadjusted peptone enrichment media made with the various peptones with the exception of Fairchild's and Parke, Davis & Co.'s (in one case). There was very scant growth in Fairchild's in the case of culture 93 (5 per cubic centimeter) and also in the case of culture 95 (350 per cubic centimeter). Cultures 400 and 404 also grew less readily in the unadjusted than in the adjusted medium. Culture 87, however, grew well in this medium at a reaction of pH 5.7. By the adjustment of the reaction of this peptone to pH 7.4, culture 93 showed 1,000,000 organisms per cubic centimeter and culture 95 showed 73,000,000 per cubic centimeter. There was no growth of culture 400 in an unadjusted Parke, Davis & Co.'s peptone (pH 5.6). In the same medium adjusted to pH 7.4 there were 8,400,000 organisms per cubic centimeter.

DIAGR. III:- GROWTH OF V. CHOLERAE, B. COLI AND B. ALKALIGENES IN PEPTONE AND ALKALINE EGG ENRICHMENT SOLUTIONS.



PEPTONE ENRICHMENT SOLUTION (REACTION UNADJUSTED).
" " " (" ADJUSTED TO pH 7.2).
ALKALINE EGG ENRICHMENT SOLUTION (REACTION UNADJUSTED).
" " " " (" ADJUSTED TO pH 9.6).

(*b*) There was a certain advantage in adjusting the reaction of all the other peptones to that of Witte's, viz, pH 7.2–7.4, inasmuch as the growth of the cholera cultures with few exceptions was increased, while *B. coli* was slightly decreased in two out of four adjusted peptones. However, three of these peptones, Squibbs's Difco's, and Armour's were close to Witte's in reaction. As has been shown in the case of other organisms which grow within rather extended limits and whose optimum is not a point, but rather a range, the adjustment of the reaction to a particular point within these limits is not of great importance.

(*c*) In the case of the alkaline medium, however, we approach the limit of the range of most active growth and come into the area of diminishing growth, particularly as far as *B. coli* is concerned. Here a slight chance of reaction may have a decided effect.

The relative amounts of growth in the egg media and the peptone solutions when planted with the cholera cultures as compared with *B. coli* planted in the same media is well shown. The cholera cultures grew almost as well in the egg media as in the peptone solutions with certain exceptions. *B. coli* on the other hand was greatly inhibited and in four out of the six unadjusted egg media there were less than 100 organisms per cubic centimeter. *B. alkaligenes* was also inhibited throughout. The results obtained with the Fairchild and Armour peptone egg media indicate that adjusting the media to a reaction of pH 9.5–9.6 is an advantage.

As a conclusion to this part of the work, we may therefore say that it is desirable to adjust the reaction of the peptone media to one slightly on the alkaline side of neutrality when the pH value is less than about pH 6.5 and that the reaction of the egg media should be adjusted to about pH 9.5 when the reaction is as acid as pH 9.0–9.2 (i. e., in cases where the two enrichment media are used conjointly).

The matter of adjusting the reaction of the egg mixture to pH 9.5–9.6 is however a rather difficult one, and would militate against the method in cholera work where one of the most important desiderata is simplicity and ease of preparation of media. The color change of the indicator thymolsulphomphthalein in the range pH 9.4 to 9.8 is slight and it is difficult to determine accurately the exact reaction. The use of the indicator thymolphthalein is also not entirely satisfactory for the reason that the color is evanescent in character. The turbidity of the egg mixture complicates very greatly the determination of accurate readings. It was therefore considered advisable to attempt adjusting the reaction by adjusting the reaction of the Dunham's solutions made of the various peptones to the reaction of Witte's, viz, pH 7.2, before the addition of the egg mixture. Owing to the different buffer properties of the various

peptones it is not to be expected that exactly the same alkaline reaction would be attained by this method. The Witte's and Squibbs's mixtures were always more alkaline than the others. In order to determine in actual practice how much difference there would be in the use of media adjusted according to the two different methods, tests were carried out with Parke, Davis & Co., Fairchild, Armour, and Difco media adjusted before and after the addition of the egg mixture. The results are shown in Table III and Diagram IV. As before the tubes of media were planted with 0.001 cubic centimeter of culture and incubated for 7 hours after which the colony count was determined by plating on glucose agar.

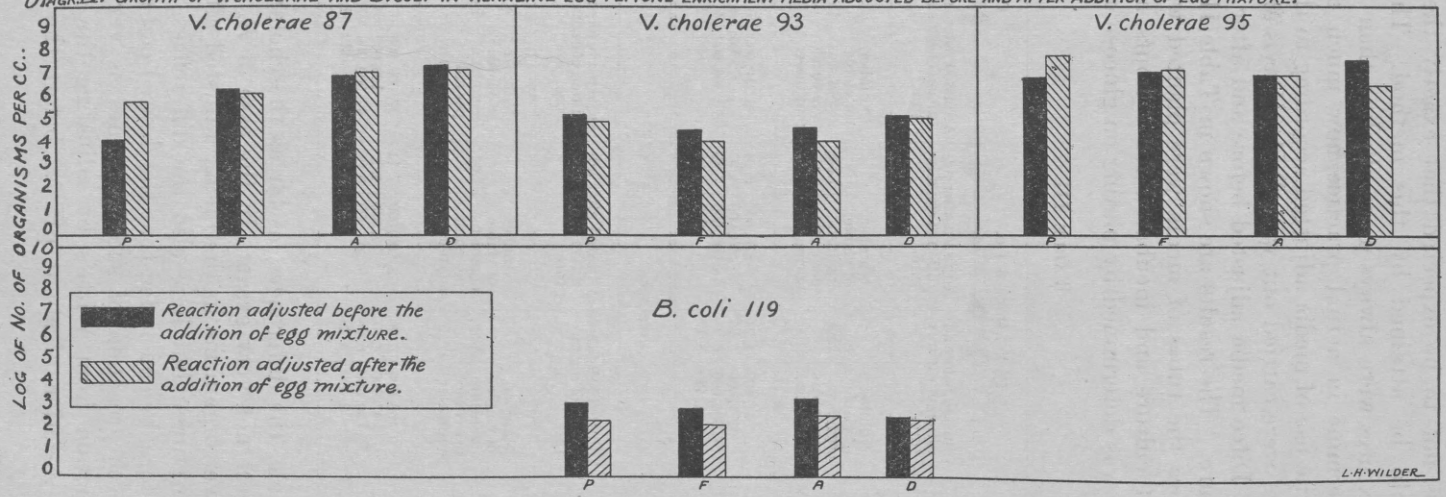
TABLE III.

Culture.	Parke, Davis & Co.				Fairchild.			
	Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.		Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.	
	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.
V. choleræ 87	17,400	4.24	870,000	5.94	2,310,000	6.36	1,690,000	6.23
V. choleræ 93	177,000	5.25	93,000	4.97	37,500	4.57	11,600	4.06
V. choleræ 95	7,900,000	6.9	78,000,000	7.89	12,300,000	7.09	14,300,000	7.16
B. coli 119	1,480	3.17	230	2.36	890	2.95	210	2.32

Culture.	Armour.				Difco.			
	Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.		Adjusted before addition of egg mixture.		Adjusted after addition of egg mixture.	
	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.	Number of viable organisms per cubic centimeter.	Log.
V. choleræ 87	9,100,000	6.96	12,800,000	7.11	27,600,000	7.44	16,200,000	7.21
V. choleræ 93	47,000	4.67	12,500	4.1	163,000	5.21	125,000	5.1
V. choleræ 95	9,600,000	6.98	9,200,000	6.96	47,000,000	7.67	4,300,000	6.63
B. coli 119	2,400	3.38	530	2.72	430	2.62	310	2.49

On the whole, the differences, as far as the cholera cultures were concerned, were not very great. In the case of *B. coli*, there were uniformly fewer organisms in the media adjusted after the addition of the egg mixtures; but here also the differences were not great. It may, therefore, be concluded that the desired end may be attained by adjusting the reaction of such peptones as vary to any great extent in reaction from Witte's by adjusting the base Dunham's solution to a reaction of about pH 7.2 before the addition of the egg mixture.

DIAGR. IV:- GROWTH OF V. CHOLERAE AND B. COLI IN ALKALINE EGG PEPTONE ENRICHMENT MEDIA ADJUSTED BEFORE AND AFTER ADDITION OF EGG MIXTURE.



3. Further information on the growth of *V. choleræ* and *B. coli* in the enrichment media was sought by planting with mixtures of the two organisms. Tubes of peptone solution and adjusted and unadjusted alkaline egg enrichment media were planted with the following combinations:

V. choleræ 87 (0.001 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

V. choleræ 93 (0.005 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

V. choleræ 95 (0.001 cubic centimeter)–*B. coli* 119 (0.1 cubic centimeter).

After 7 hours incubation the growth was streaked on Endo plates to determine the relative proportion of the two kinds of colonies. The results with culture 95 are shown in Table IV.

TABLE IV.—*Peptone enrichment and alkaline egg peptone enrichment solution planted with V. choleræ 95 (0.001 cc) and B. coli 119 (0.1 cc) streaked on Endo plates.*

	Alkaline egg enrichment solution.				Peptone enrichment solution.	
	Reaction unadjusted.		Reaction adjusted to pH 9.5.		Reaction unadjusted.	
	Number of *V. choleræ*.	Number of *B. coli*.	Number of *V. choleræ*.	Number of *B. coli*.	Number of *V. choleræ*.	Number of *B. coli*.
Parke, Davis & Co.'s peptone:						
Plate 1	Numerous	550	Numerous	75	Colonies indistinguishable.	Numerous.
Plate 2	350	38	650	0	75	500.
Plate 3	48	3	42	1	0	191.
Witte's peptone:						
Plate 1	550	12	No adjusted medium.		Colonies indistinguishable.	Numerous.
Plate 2	1	0			3	750.
Plate 3	0	0			1	42.
Fairchild's peptone[a]						
Plate 1	Numerous	Numerous	Numerous	250	Numerous	Numerous.
Plate 2	330	250	135	14	390	620.
Plate 3	35	26	19	7	65	125.
Armour's peptone:						
Plate 1	1,200	238	Numerous	75	Colonies indistinguishable.	Numerous.
Plate 2	0	0	78	1	16	120.
Plate 3	0	0	0	0	12	112.
Difco peptone:						
Plate 1	1,200	210	1,500	450	Numerous	Numerous.
Plate 2	35	115	350	42	127	550.
Plate 3	5	9	8	2	0	33.
Squibb's peptone:						
Plate 1	Numerous	25	No adjusted medium.		Colonies indistinguishable.	Numerous.
Plate 2	208	3			3	750.
Plate 3	9	0			1	42.

[a] Reaction of peptone enrichment medium adjusted to pH 7.4.

The advantage of using the alkaline egg medium for enrichment of *V. choleræ* is indicated by the greater number of colonies of *V. choleræ* and the small number of *B. coli* colonies occurring on the plates streaked with these cultures as contrasted with the small number of *V. choleræ* and large number of *B. coli* streaked with the peptone solution cultures. The results obtained with the adjusted alkaline egg peptone solution also are more favorable for the isolation of *V. choleræ* than the unadjusted egg medium.

The results with cultures 87 and 93 were similar. Colonies of 93 were indistinguishable on all of the plates streaked with the peptone solution cultures except in the case of Parke Davis peptone.

4. Alkaline egg agar medium. Tests were carried out to determine what was the reaction of Witte's alkaline egg agar favorable for the growth of *V. choleræ* and inhibitory to *B. coli*. The medium was made according to the Goldberger formulæ. One portion of agar base was left unadjusted, another was adjusted to pH 7.0, and a third portion was adjusted to pH 7.4 before the addition of the egg mixture. The plates were streaked with cholera cultures 87, 93, 95, and 97 and *B. coli* 119. The results are shown in Table V.

TABLE V.—*Growth of V. choleræ and B. coli on Witte's alkaline egg agar.*

Reaction of agar base.	*V. choleræ* 87		*V. choleræ 93.*		*V. choleræ 95.*		*V. choleræ* 97.		*B. coli 119.*	
	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.
Unadjusted pH, 5.8	650	[1] 90	580	55	880	75	650	110	[2] 850	22
Adjusted pH, 7.0	850	85	254	55	740	74	680	110	[3] 0	0
Adjusted pH, 7.4	920	90	655	55	870	65	830	90	[4] 0	0

[1] Divisions of micrometer scale (50=1 millimeter).
[2] Distinct colonies.
[3] Several areas of hazy growth. Very minute pin-point colonies.
[4] No growth.

By adjusting the reaction of the agar base to pH 7 before the addition of the egg mixture a medium was obtained which almost completely prevented the development of *B. coli*. By adjustment to pH 7.4 no growth whatever was visible. The cholera colonies were, however, as numerous and as large as on the unadjusted plates.

5. An adjustment of the agar base to a reaction in the neighborhood of pH 7.4 was, therefore, in accordance with the above results, considered a favorable one and tests were then carried out with the various other peptones, and comparisons obtained of growth on unadjusted agar and agar adjusted to the above reactions. The results are presented in Table VI.

TABLE VI.—*Growth of V. choleræ, B. coli and B. alkaligenes on unadjusted and adjusted alkaline egg agar.*

	pH of agar base.	*V. choleræ* 87.		*V. choleræ* 90.		*V. choleræ* 93.	
		Number of colonies.	Size.[a]	Number of colonies.	Size.	Number of colonies.	Size.
Parke, Davis & Co.'s:							
Unadjusted	5.8	680	140	410	150	495	90
Adjusted	7.4	550	150	232	160	450	65
Witte's:							
Unadjusted	6.4	116	100	610	125	178	17–60
Adjusted	7.4	352	125	350	125	112	11–60
Fairchild's:							
Unadjusted	5.4	750	130	400	150	44	15–90
Adjusted	7.4	1,200	130	750	110	0	------
Armour's:							
Unadjusted	6.4	79	150	334	140	198	20–70
Adjusted	7.4	145	170	97	160	129	20–50
Difco:							
Unadjusted	6.4	500	100	72	160	129	15–80
Adjusted	7.4	540	130	39	160	62	10–70
Squibb's:							
Unadjusted	6.6	680	125	236	160	[b] 0	15–65
Adjusted	7.4	219	140	204	160	[c] 0	30

	pH of agar base.	*V. choleræ* 95.		*V. choleræ* 400.		*B. alkaligenes* 116.		*B. coli* 119.	
		Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.	Number of colonies.	Size.
Parke, Davis & Co.'s:									
Unadjusted	5.8	1,100	105	630	120	[d]	[e]	1,250	30
Adjusted	7.4	410	115	850	90	0	------	0	------
Witte's:									
Unadjusted	6.4	720	110	144	110	0	------	126	30
Adjusted	7.4	750	90	410	110	0	------	0	------
Fairchild's:									
Unadjusted	5.4	760	125	30	130	24	22	1,500	40
Adjusted	7.4	630	90	45	130	0	------	0	------
Armour's:									
Unadjusted	6.4	435	110	125	150	[f] 0	8	125	18
Adjusted	7.4	224	75	480	120	0	------	0	------
Difco:									
Unadjusted	6.4	390	100	720	125	0	------	8	11
Adjusted	7.4	56	110	1,200	125	0	------	0	------
Squibb's:									
Unadjusted	6.6	890	110	560	150	0	------	80	22
Adjusted	7.4	730	105	830	125	0	------	0	------

[a] Divisions of micrometer 50=1 millimeter.
[b] 117 on first plate.
[c] 13 on first plate.
[d] Numerous.
[e] Pin point.
[f] 89 on first plate.

The advantage of adjusting the agar to a reaction of pH 7.4 is shown by comparing the results obtained with *B. coli* on adjusted and unadjusted media. In all cases there was a growth on the unadjusted plates, and none on the adjusted plates. The size of the colonies of *B. coli* on the unadjusted media was small in comparison with the size of the cholera colonies and would perhaps not interfere with the isolation of *V. choleræ*, but the advantage of adjusting the medium to a more alkaline reaction is nevertheless evident.

6. Isolation of *V. choleræ* from artificially contaminated stools.

Media for the isolation of the organism were prepared in accordance with the information obtained in the above tests. Peptone

enrichment media were unadjusted with the exception of Fairchild's and Parke, Davis & Co.'s, which were adjusted to pH 7.4. The alkaline egg enrichment media were prepared by adjusting the base Dunham's solution to a reaction of pH 7–7.2 and the agar adjusted to about pH 7.4 before the addition of the egg mixture.

A sample of stool was emulsified, strained through sterile gauze and divided into several parts, one of which was left uncontaminated to serve as control and two other portions were contaminated with cholera cultures as follows:

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 87.

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 95.

4 cubic centimeters feces emulsion—1 cubic centimeter broth culture *V. choleræ* 400.

0.1 cubic centimeter of the mixtures were planted in the two enrichment media, incubated for 7 hours and streaked on the alkaline egg agar plates. The results obtained with cultures 95 and 400 are indicated in Table VII. Similar results were obtained with culture No. 87. Efforts to isolate cholera culture No. 93 were not as successful. By using a comparatively large amount of the culture, it was, however, isolated from Parke, Davis & Co., Witte, and Difco media. No colonies which could be identified as *V. choleræ* were fished from plates made with Fairchild, Armour, or Squibb's peptones. This organism seems to be an aberrant one in some respects and throughout this work always failed to grow as readily as the other organisms employed.

TABLE VII.—*Alkaline egg agar—Isolation of V. choleræ from contaminated stools.*

	Peptone enrichment solution.				Alkaline egg enrichment solution.[a]			
	V. choleræ 95.		Other colonies.		*V. choleræ* 95.		Other colonies.	
	Number.	Size.	Number.	Size.	Number.	Size.	Number.	Size.
Parke, Davis & Co.	625	85	92	24	465	85	164	24
Witte	(b)	50	(c)	------	475	65	105	16
Fairchild	[d] 1,200	65	55	35	111	74	175	46
Armour	[d] 1,200	52	46	18	132	53	196	44
Difco	[d] 1,000	46	25	16	220	56	82	37
Squibb's	[d] 1,500	54	13	17	178	67	72	21
	V. choleræ 400.		Other colonies.		*V. choleræ* 400.		Other colonies.	
	Number.	Size.	Number.	Size.	Number.	Size.	Number.	Size.
Parke, Davis & Co.	265	100	62	60	136	110	61	70
Witte	460	65	0	------	525	90	95	45
Fairchild	18	90	325	60	53	100	124	75
Armour	335	110	82	70	44	90	150	50
Squibb's	485	110	135	55	315	120	53	50

[a] Witte's and Squibb's peptones required no adjustment in the enrichment media.
[b] Very numerous.
[c] Not distinguishable.
[d] Number estimated.

Considering Table VII, which represents an application of the results obtained in the previous work, it appears that the results with Witte's peptone were somewhat more favorable than those obtained with the other peptones in that on those plates streaked with the growth in peptone enrichment solution the *B. coli* colonies were indistinguishable or absent and the number of cholera colonies was very large. In no case, however, was there any difficulty in distinguishing cholera colonies on the other plates, as they were usually more numerous and larger than the other colonies. The cholera colonies are more transparent and darker in color than those of *B. coli*, which are white and opaque. In all the previous work no particular advantage had been observed in the use of Witte's peptone over the other peptones, except for the fact that this peptone, together with Squibb's, were sufficiently alkaline to require no adjustment of reaction in the alkaline egg enrichment fluid.

In the test with culture 95 the results with the peptone solution apparently are more favorable than with the alkaline egg enrichment medium. The amount of contamination of *V. choleræ* was comparatively large, and smaller amounts would probably show more the advantage of the alkaline egg enrichment fluid. Such a test, in which the contamination with the cholera organism was one-tenth the volume of feces emulsion instead of one-fifth, showed equally good results on plates made from the two enrichment fluids, all the plates showing almost pure cultures of *V. choleræ*, except in the case of Fairchild's where the cholera colonies and the other colonies were about equally divided.

SUMMARY.

In conclusion it may be stated that the American peptones in general are suitable for the isolation of *V. choleræ*. Good growth was obtained in all media with the exception of Fairchild's peptone solution and sometimes Parke, Davis & Co.'s peptone solution, which were found to be too acid. These by adjustment to a more alkaline reaction could be made favorable.

The results of this work do not show any great advantage of one particular peptone over the others.[5] The Parke, Davis & Co. peptone when properly adjusted, appeared to furnish more luxuriant growths than some of the others, perhaps on account of suitable amino acids. Squibb's peptone offers the advantage of a reaction as alkaline as Witte's, and less adjustment of reaction was necessary than in the

[5] The tests reported in this paper were carried out with one peptone of each manufacturer, except in the case of Parke, Davis & Co. in which three were used. The peptones used were received at the laboratory two or more years before the date of this paper. It was not possible to carry out tests with a number of peptones of each manufacturer. The results obtained should not therefore necessarily be interpreted as applying to all the peptones of one manufacturer, as it is recognized that different lots of peptones from the same manufacturer vary to some extent.

case of some of the other peptones. Good results were obtained with Difco peptone, which was only slightly more acid than Witte's. Armour's peptone throughout showed a tendency to favor *B. coli* somewhat more than those just enumerated. Fairchild's peptone appeared on the whole to be least suitable in that it was found to be more acid than the others and apparently some other elements were lacking even though the reaction was properly adjusted.

The general statement may be made in regard to reaction that owing to the variation among different peptones it is advisable to adjust to a more nearly uniform reaction, particularly in the case of those which vary to any considerable degree from Witte's in reaction.

If the two enrichment media are used conjointly it does not appear to be of any great advantage to adjust the reaction of the peptone solution to a definite point if the reaction is somewhere near neutrality. If the peptone solution alone is used for enrichment it would seem advisable to adjust to a rather alkaline reaction, though it can not be expected that a stable medium will be obtained without a buffer if the reaction is made more alkaline than about pH 8.

By adjusting the reaction of the Dunham's solution in the alkaline egg enrichment medium and of the agar in the alkaline egg plating medium to a point slightly on the alkaline side of neutrality, about pH 7.2–7.4, before the addition of the egg mixtures, suitable media are obtained. Adjustment of the reaction to this point is simple and definite, whereas adjustment to a uniform reaction after the addition of the egg mixture is too difficult and uncertain to be practical.

Most laboratories at the present time are equipped to adjust the reaction of culture media by the new method, and if the adjustment of the agar and Dunham's solution is made at the time of preparation there will be no unnecessary delay at the time the medium is to be used.

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